CHARACTERIZATION OF KININ ACTION AND KININ RECEPTORS IN CENTRAL NERVOUS SYSTEM TISSUE

BY

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To Mom and Dad

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KEY TO ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ADH	antidiuretic hormone
B_{max}	maximum number of sites bound
cm	centimeter
cpm	counts per minute
CSF	cerebrospinal fluid
DMEM	Dulbecco's modified Eagle medium
FBS	fetal bovine serum
fmoles	femtomoles
g	gram or acceleration due to gravity
HPLC	high pressure liquid chromatography
HS	horse serum
IC_{50}	concentration which inhibits 50% of maximum value
ivt	intracerebroventricular
K_D	equilibrium dissociation constant
K_m	Michaelis-Menten constant
KIU	kallikrein inhibitory unit
Lys	lysine
mCi	millicurie
Met	methionine
ml	milliliter

mmHg	millimeters of mercury
µCi	microcurie
µg	microgram
µl	microliter
µM	micromolar
µmole	micromole
ng	nanogram
nM	nanomolar
nmoles	nanomoles
pg	picogram
pM	picomolar
pmoles	picomoles
R_f	distance of substance migration divided by distance of solvent front migration
w/v	weight to volume ratio

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION OF KININ ACTION AND KININ RECEPTORS IN CENTRAL NERVOUS SYSTEM TISSUE

by

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Kinins are nine to ten amino acid peptides cleaved from kininogen by kallikrein. The ability of kinins injected into the brain to induce changes in blood pressure and the ability of kinins to bind to specific, high affinity recognition sites in rat brain cell culture were examined.

The central pressor response in rats to kinins was studied by intracerebroventricular (ivt) pretreatment with different pharmacological agents. Saralasin and Captopril potentiated, and indomethacin (ivt) attenuated, the central kinin pressor response. These results suggest that the centrally mediated pressor response to ivt kinins is modified by an inhibitory action of the brain renin-angiotensin system, and that prostaglandins may be formed as intermediaries is this kinin-induced response.

The central kinin pressor response was localized to the ventral third ventricle by cream plugs which block drug access to discrete

regions of the ventricular surface. Ventral third ventricle plugs blocked the pressor response to ivt bradykinin, while fourth ventricle plugs did not.

The possibility that kinins bind to specific, high affinity recognition sites in brain tissue was examined. Rat brain cell culture provided a system whereby nonspecific binding and degradation of the ligand could be minimized. Using the iodinated ligand ^{125}I-Tyr-bradykinin, binding was observed to be time and pH dependent. Scatchard analysis of saturation experiments yielded two components with dissociation constant and maximum binding site concentration averaging 1 nM and 100 fmoles/mg protein, and 16 nM and 1000 fmoles/mg protein, respectively. The binding sites were specific for kinins and kinin analogues, and the order of potency in competing for ^{125}I-Tyr-bradykinin binding was Lys-bradykinin > bradykinin > Tyr-bradykinin > Tyr^{8}-bradykinin >>> Des-Arg^{9}-bradykinin. Monovalent and divalent cations inhibited kinin binding. Comparison of competition curves performed in glial-enriched versus neuron-enriched cultures suggested that the kinin binding sites resided primarily on neurons. These data enhance the existing evidence suggesting kinins as neurotransmitters or neuromodulators.

CHAPTER I
INTRODUCTION

A Brief History of Kinins and Kinin Research

The roots of kinin research reach back to 1909 with the observation by two French surgeons that intravenous injections of fractions extracted from urine resulted in a transient fall in blood pressure (1). This hypotensive effect was attributed by Frey and Kraut (2) to a thermolabile, nondialysable substance they termed "Kreislaufhormon" or essentially "circulating hormone." Search for the tissue of origin led to the discovery of a hypotensive factor in pancreas which was given the name kallikrein (3) derived from the Greek word for pancreas, kallikreas. Werle and colleagues (4) observed the inactivity of kallikrein on isolated guinea pig intestine but discovered that a marked increase in activity occurred following preincubation of the substance with blood. Kallikrein was postulated to cause the formation of an intestinal contracting substance which was eventually termed kalladin (5). The following year, 1949, Rocha e Silva and co-workers (6) reported their observation of a factor formed in the blood upon incubation with trypsin which contracts the isolated guinea pig ileum. Because the contractile response was slow relative to that induced by histamine, the factor was named bradykinin, derived from the Greek bradys kinein meaning "slow to move." Eventually bradykinin was shown to be a peptide (7) and purified to homogeneity (8). In 1960 Boisonnas et al. (9) reported the correct amino acid sequence and successfully synthesized the peptide.

Subsequently, the biologically active kinins kalladin, Lys-bradykinin, and Met-Lys-bradykinin were isolated and purified (10, 11). With the availability of the synthetic peptides, work on the biological actions of kinins extracted from various sources could be confirmed and consolidated.

The discovery by Ferreira and Vane (12) in 1967 that kinins were rapidly degraded to inactive peptides upon passage through the lung placed in doubt any idea that bradykinin might be a circulating hormone. Based on this observation Rocha e Silva (13) suggested that kinins might be better thought of as "tissue" hormones (autocoids). Classically a hormone is defined as a substance formed in one part of the body and carried via the blood to another organ or region where the substance acts. A "tissue" or local hormone varies from this classical definition in that it is formed at the site of action.

Numerous investigations have led to the uncovering of a variety of biological actions elicited by kinins on peripheral tissues. Four biological activities characterize kinins: contraction of nonvascular smooth muscle (except for rat duodenum), vasodilation, increased vascular permeability, and pain production (for a review see 14). Kinins have been suggested as regulators in local circulatory control (15), fibrinolysis and clotting (16, 17), inflammation (18), control of electrolyte and water excretion by the kidney (19), metabolic and mitotic activity of different cell types (20-22) and modulators within the nervous system (23), as well as other physiological and pathological states. Nevertheless, the kallikrein-kinin system, though implicated, has yet to be conclusively demonstrated as an active participant in any

physiological process. Several technological insufficiencies may explain, at least in part, this deficiency. Until recently (24) one problem has been lack of a sensitive radioimmunoassay for plasma kinins. It is difficult to raise an antibody to bradykinin with little or no crossreactivity to kininogen. One must also be able to insure against the de novo formation of kinins during sample preparation. Consequently, accurate measurement of kinin levels has been difficult. Another technological shortcoming which is currently being corrected (25, 26) is the development of an adequate assay for probing kinin-receptor interactions. Both RIA and radioligand binding are necessary to complement bioassays which may be subsensitive to peptide concentrations in the picomolar range. Finally, the study of kinin action lacks an adequate antagonist, a fundamental component to the study of any putative physiological system. It is through these modes of attack that investigators are approaching current problems in kinin research.

The Kallikrein-Kinin System

Perhaps the most useful means of understanding the kallikrein-kinin system is by dissecting it into its component parts. An outline of this system is in Figure 1.

Kallikreins

Kallikreins can be roughly divided into two categories, plasma and glandular. Kininogens are their only known natural substances. Plasma kallikreins, by definition, are found in blood, whereas glandular kallikreins have been isolated from tissues. The two enzymes are immunologically (27,28) and enzymatically distinct. Plasma kallikreins

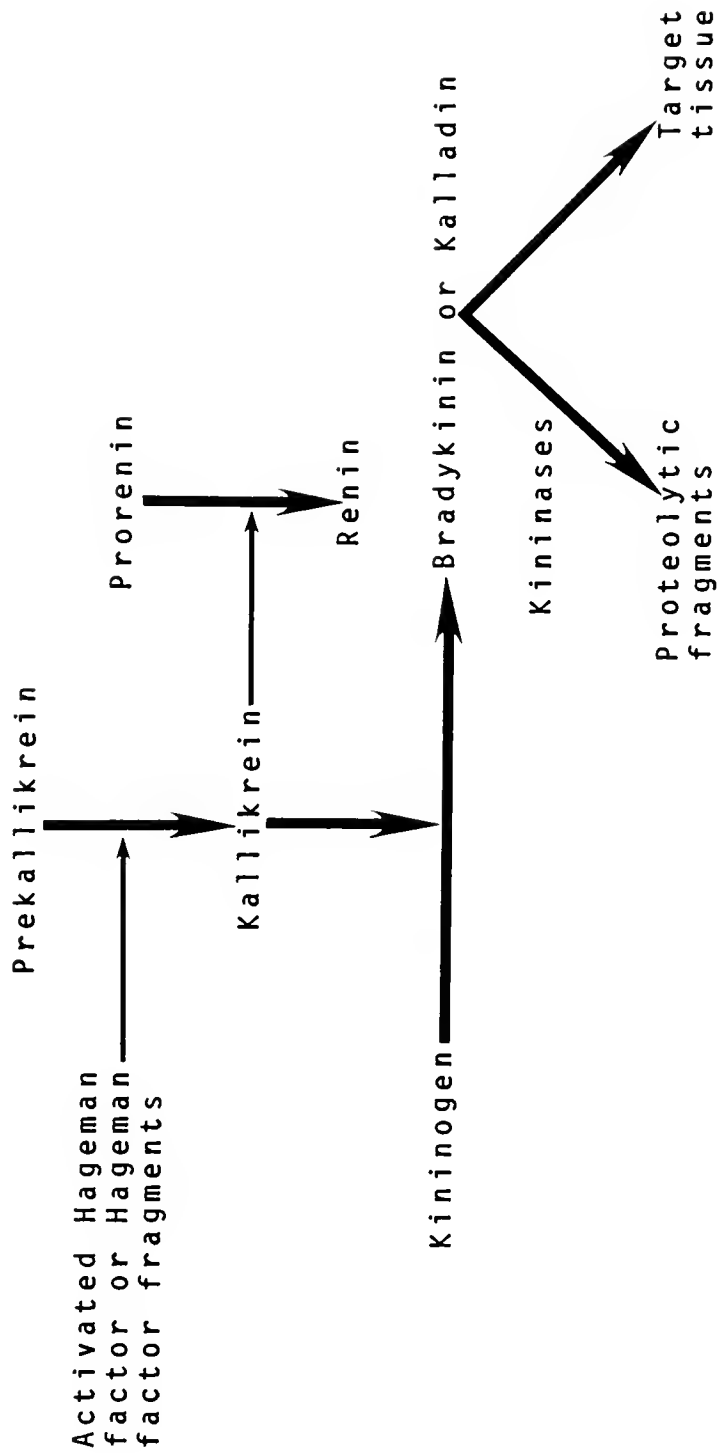


Figure 1. The basic cascade for kinin production.

cleave high molecular weight kininogens to form bradykinin, while glandular kallikreins enzymatically release kalladin (Lys-bradykinin) (12, 29). Moreover, soybean trypsin inhibitor inhibits plasma kallikreins but not glandular kallikreins (30). Plasma prekallikreins, generated in the liver (31), can be activated by Hageman factor (32) or trypsin (33). Activation yields the functional serine protease (34). Human plasma kallikrein is 108,000 daltons as measured by sodium dodecyl sulfate disc gel electrophoresis (32, 33), and has demonstrated several peaks between pH 7.7 and 9.4 during isoelectric focusing. Plasma kallikreins from other animals have similar molecular weights of around 100,000 (35).

Glandular kallikreins, which occur mainly in the pancreas, salivary glands and kidney, as well as in their exocrine secretions, are glycoproteins of 25,000 to 40,000 molecular weight (36, 37) and isoelectric points near 4.0 (37). There is also heterogeneity associated with distinct glandular kallikreins. Habermann separated pig pancreatic kallikrein into two forms, A and B, by electrophoresis (38).

Glandular kallikreins are immunologically identical. Antibody to human urinary kallikrein forms lines of identity with human salivary and pancreatic kallikrein as well as with kallikrein exuded in human sweat (39). Urinary kallikrein is generally believed to be identical to renal kallikrein. The biochemical properties of the two enzymes are essentially indistinguishable (40).

Kallikreins can be assayed by several different methods. Bioassay, the assessment of kallikreins' ability to release kinins from plasma substrate, is one method of assay. The amount of kinin liberated is

measured by its ability to contract smooth muscle preparations (e.g., guinea pig ileum or rat uterus), or to lower blood pressure by decreasing vascular resistance. Difficulties arise, however, unless care is taken to assure that samples are free from contaminating proteases with kininogenase activity and that all kininase activity is blocked to prevent diminution of the oxytocic or vasodilatory effects.

Alternatively, assays have been developed to take advantage of plasma and glandular kallikreins' ability to hydrolyze esters of α-N-acylated arginine. Examples of synthetic substances are α-N-tosyl-L-arginine methyl ester (TAMe), benzoyl-L-arginine ethyl ester (BAEe) and benzylcarboxyl-L-arginine ethyl ester (CBZ-AEe). Hydrolysis of these substrates can be followed spectrophotometrically, and titrimetrically or radiolabelled substrates can be used and the hydrolysis products separated and counted (41). These assays can reveal unusual characteristics such as time dependence of enzymatic activity (42). However, esterase activity does not parallel activity in bioassay or radioimmunoassay.

Radioimmunoassays (RIA) using specific antibodies to plasma or glandular kallikreins have been developed (24, 27). A specific RIA eliminates the need to purify kininogens or inhibit kininases, and assures greater specificity than a bioassay.

Kallikreins can be inhibited by several substances. Natural inhibitors are C1 esterase inhibitors (43), soybean trypsin inhibitor (plasma kallikrein only) (30) and bovine pancreatic trypsin inhibitor (Trasylol, Aprotinin) (44) which has been identified in several bovine organs. The major synthetic inhibitor is di-isopropyl-fluorophosphate

(DFP) which has been used to demonstrate the serine protease activity of kallikreins (45).

Plasma kallikrein is localized in the blood. It may be complexed endogenously to α_2-macroglobulin which allows the enzyme some protease activity while preventing binding of circulating inhibitors (e.g., C1 esterase inactivator). Glandular kallikrein has localized to various cell types depending on the tissue studied. In rat submandibular gland kallikrein immunoreactivity has been observed in the granular tubules, striated ducts and some main duct cells, but not acinar cells (46, 47). Subcellularly, the salivary gland has been suggested to be localized to zymogen granules (48, 49). The cellular localization of pancreatic kallikrein (acinar cells or beta cells) has continued to be debated (50, 51). While the discrepancy is still unresolved, efforts have been made to explain the conflicting results (52).

Much work has been done on the cellular localization of renal kallikrein (53). A recent report utilizing radioimmunoassay demonstrated the highest kallikrein content in the outer cortex with progressively decreasing immunoreactivity toward the papillary tip (54). Microdissected cortical nephrons had their highest kallikrein content in the connecting tubule followed by the initial collecting tubule and the distal convoluted tubule. Little or no kallikrein was identified in glomeruli, proximal convoluted tubule, proximal straight tubule or cortical thick ascending limbs (54).

Functionally, plasma kallikrein can cause the formation of bradykinin potentially leading to changes in local vascular tone, and can potentiate Hageman factor activation (55) leading to the clotting

cascade. Glandular kallikreins may have physiological roles in local circulatory regulation (15) and control of electrolyte and water balance by the kidney (19).

Kininogens

Kininogens, formed in the liver (56), are glycoproteins that can be categorized by their molecular weight and susceptibility to enzymatic cleavage. Low molecular weight (LMW) kininogen has been reported to be between 50,000 (57) and 78,000 (58) daltons, and its isoelectric point is 4.7 (57). LMW kininogen has been resolved into two types: Type I (32% of LMW kininogen) loses its kinin generating ability upon extensive treatment with carboxypeptidase B; Type II (68% of LMW kininogen) is insensitive to carboxypeptidase B. These observations were interpreted as indicating the kinin moiety resides on the C-terminal of LMW kininogen I, but is buried within the Type II molecule (59).

Human high molecular weight (HMW) kininogen is between 108,000 and 120,000 daltons (58, 60). HMW and LMW kininogen are immunologically similar (61); however, plasma kallikrein prefers HMW kininogen as a substrate whereas glandular kallikrein can form kallikrein from either substrate (62, 63).

Kinins

The primary mammalian kinins, bradykinin and kalladin (Lys-bradykinin), are nona- and decapeptides, respectively (Figure 2). The arginine residues and additional lysine in kalladin make these peptides very basic. Kinins are typically found in pg/ml quantities in the blood (64).

Delineation of such low levels, however, requires a sensitive radioimmunoassay, and there are problems inherent to radioimmunoassay of kinins (65, 66). Among these problems are the poor immunogenicity of kinins and the frequent observation that kinin antibodies cross-react with kininogens. Another difficulty which must be carefully controlled for is prevention of kinin formation or degradation during the collection of biological fluid. Bioassays have been used for determination of kinin levels but the problems of sensitivity and specificity are the same for kinins as they are for other substances.

The biological actions of kinins have been listed above. A direct physiological role for kinins has not been determined. However, they have been suggested as playing roles in clotting and fibrinolysis (16); sodium retention by the kidney (67), vasodilation in exocrine glands (15) and the heart (68); uterine contraction (69); sperm-motility (21); catechol release by the adrenal (70); ovulation (71); and cardiovascular regulation by the brain (72). Pathologically, kinins have been suggested for roles in allergy (73), arthritis (74), carcinoid and "dumping" syndromes (75, 76), headache (77), hypertension (78) and inflammation (79).

Kininases

Some proteolytic enzymes which can degrade bradykinin are shown in Figure 2 along with their sites of cleavage. An aminopeptidase has been isolated from blood (29, 80) of approximately 95,000 daltons which can cleave lysine from the amino terminal of kalladin, but which will not cleave the Arg^1-Pro^2 bond of bradykinin. Trypsin will also cleave lysine from kalladin, but under normal conditions it is not active in

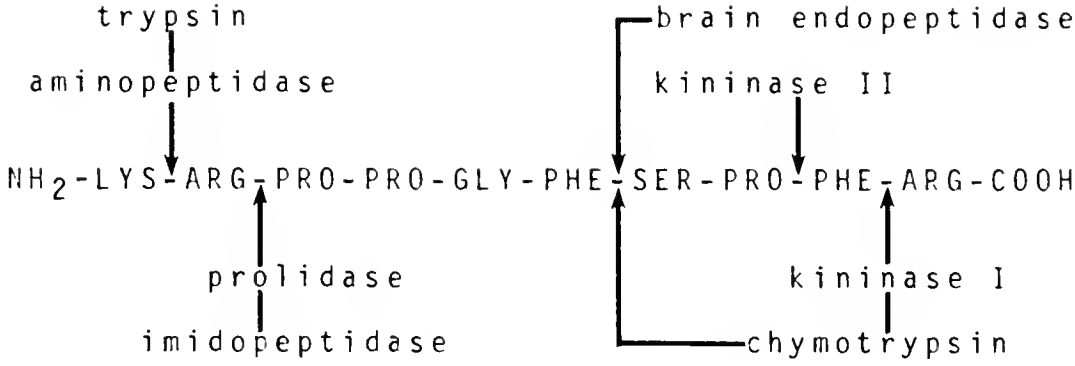


Figure 2. Primary structure of kalladin (lys-bradykinin) and bradykinin. Site of action of known proteolytic enzymes.

blood (81). Except for indirect evidence provided in one report (82) prolidase and imidopeptidase are believed to be intracellular constituents (80, 83). Consequently highly charged molecules like kinins would not be expected to be degraded by these enzymes unless specific transport systems or receptor-mediated endocytosis shifted the peptide to an intracellular compartment. In general, degradation of kinins at the amino terminal is not believed to play a major role in the circulation.

Enzymes with specificity toward the carboxy terminal of kinins are considered the most important in degrading these peptides. Primary among these peptides are kininase I and kininase II. Kininase I, synonymous with carboxypeptidase N, is responsible for 90% of the degradation of kinins in plasma (84, 85). It is a circulating carboxypeptidase which can cleave the C-terminal arginine from bradykinin and kalladin (86). Kininase II has been shown to be identical to angiotensin converting enzyme (87, 88). Kininase II is a membrane bound enzyme with a wide distribution. It has been identified in various vascular beds (89, 90), kidney (91), testicular tissue (92), brain (93, 94) and choroid plexus (95). The enzyme has specificity for the Pro^7-Phe^8 and Phe^5-Ser^6 bonds of bradykinin (88).

Bradykinin is an excellent substrate for kininase II. The K_m for degradation of bradykinin is lower than the K_m for angiotensin I (96). Kalladin is not as susceptible to kininase II as bradykinin (97). Kininase II has been identified as glycoprotein with a wide molecular weight range (129,000 to 480,000) (88, 89) due primarily to the variability of its carbohydrate moiety (88).

Both kininase I and kininase II are metalloproteins as indicated by their inhibition with 1,10-o-phenanthroline or other metal chelators (98). Other natural and synthetic inhibitors of kinin degradation have been discovered. The venom of Bothrops jararaca has been shown to contain peptide inhibitors whose isolation leads to the subsequent synthesis of similar peptides with enhanced abilities to inhibit kinin degradation (99). Other synthetic chelators that inhibit kininase II, such as SQ 14,225 (Captopril), have also been developed (100).

Chymotrypsin and an endopeptidase described in rabbit brain can also degrade kinins (101, 102). Chymotrypsin can split the C-terminal residue or the Phe^5-Ser^6 bond (102). The Phe^5-Ser^6 bond is also a site of cleavage by the brain endopeptidase (101).

Circulating kinins are kept at a low level by both low rates of production and rapid degradation. Kininase I and II are the major enzymes in the blood. If kinins are formed discretely in various organs (11), the mode of degradation may be quite different than that observed in blood.

Kinin Action in the Central Nervous System

Study of the biochemistry and physiology of kinins has centered around their properties and actions in peripheral tissue. However, kinins may also directly influence the actions of the central nervous system (CNS) either by entering from the periphery (103), or by being synthesized within CNS structures by an endogenous kallikrein-kinin system (104, 105). One approach to studying the central biological properties of a substance is by injecting the substance into the brain (106). If the dose injected into the brain is lower than the dose which

is effective peripherally, or if the response under observation is opposite to that seen peripherally, it can be ascertained that the substance is acting on central structures. Additionally, electrophysiology can be useful to show that a particular substance can modify the electrical activity of brain cells. However, evidence demonstrating that a substance is part of an endogenous system in the brain requires both physiological and biochemical applications.

Brain Kininogenases

Kallikrein-like activity has been isolated in the microsomal fraction of rabbit brain (107, 108). Furthermore acetone treatment (which activates prekallikrein) increases kallikrein-like activity in these homogenates. Shikimi et al. (109) determined that kallikrein-like activity in brain was greatest in the cerebral cortex, but lowest in brain stem.

Kinin-Like Activity in Brain

Correa et al. (104) are the only investigators to immunocytochemically document the presence of kinin-like antigens in brain tissue. All positively staining cells were localized to the hypothalamus, whereas positively staining fibers were more widespread. Fibers were observed in periaqueductal gray matter, hypothalamus, lateral septal region, perirhinal and singulate cortex and ventral portions of the caudate-putamen. Low levels (5 pmoles/g brain) of kinin-like material have also been measured in brain by RIA (105). Hori (107) reported the presence of a partially purified kinin-like peptide from rabbit brain which had similarities to bradykinin in bioassay and electrophoretic mobility. Similarly, Pela and colleagues (110) also extracted kinin-like activity from rabbit brain.

Brain Kininases

Kininase activity in brain has been described in rat (109), mouse (111), rabbit (107, 112), dog (113), cat (113) and guinea pig (114). The majority of kinin degrading activity appears in the soluble fraction during subcellular fractionation (107, 112).

While kininase II is found abundantly in brain (94) it is certainly not the sole inactivator of kinins in the brain. Kininases distinct from kininase II have been described by Carmargo et al. (101), Marks and Pirotta (115) and Oliveira et al. (116) in brain tissue. Nevertheless, the high concentration of kininase II observed in choroid plexus (94) is probably the primary cause for the rapid degradation of kinins injected into the brain ventricles (105).

Biological Effects of Central Bradykinin

The biological actions of bradykinin which are mediated through the CNS have been studied primarily by injecting the peptide into the brain or into blood vessels which perfuse the brain. Of the various biological effects that bradykinin elicits through the CNS, the most striking is a sustained rise in blood pressure (72, 117, 118). The central pressor response is in direct contrast to the short-lived but potent vasodilation observed when the peptide was injected into the peripheral circulation.

The characteristics of the central pressor response varied in different laboratories. Generally, anesthetized animals receiving bradykinin via intracarotid or intracerebroventricular (ivt) injections have demonstrated biphasic pressor responses--a short depressor effect followed by a sustained pressor response (103, 119, 120). This biphasic

response did not appear to be the result of a direct action of bradykinin on the vasculature as it persisted in animals whose cerebral blood flow was separated from the peripheral circulation in a cross-perfused preparation (103). The initial fall in blood pressure has been suggested to be mediated through activation of α-adrenergic vasodepressor mechanisms in supramedullary centers (103, 120).

Conscious and free-moving animals typically elicit only a pressor response when bradykinin is injected centrally (117, 118, 121), although an exception has been observed (119). This pressor response to ivt bradykinin is believed to be mediated through prostaglandins (103, 121, 122) as well as noradrenergic systems (72, 103, 117). Pain production resulting in an elevation of blood pressure has been discounted by the observation that intravenous analgesics do not block the central pressor response (123, 124). Interestingly, centrally administered bradykinin has been reported to have an antinocioceptive effect of its own (124).

Consistent with the suggestion that the central action of kinins on blood pressure is mediated through noradrenergic systems (72, 103), Capek et al. (125) observed a 40% reduction in norepinephrine one hour after ivt injection of 1-5 μg of bradykinin. However, work with bradykinin effects on dopamine (123, 126, 127) and serotonin (123, 127) are equivocal and no clear conclusion can be drawn about ivt kinin effects on these amines. Bradykinin has also been suggested to release vasopressin and ACTH from the pituitary. Median eminence lesions abolished the urine-concentrating effects of ivt bradykinin (118). Similarly, intracarotid bradykinin elevated coricosterone levels (used as an index of ACTH release) and this effect was abolished by

hypophysectomy (128). However, an effect of bradykinin causing secondary, vasopressin-induced, release of ACTH cannot be ruled out by the above.

A hyperthermic response to kinins has been observed in rabbits (129). Bradykinin, kalladin, and Met-Lys-bradykinin were equivalent in elevating rectal temperature, whereas Des-Arg^9-bradykinin had no effect. The hyperthermia was antagonized by acetominophen and indomethacin suggesting prostaglandin synthesis as a mediating event in the response.

Electrophoretic studies with kinins on brain tissue have been carried out. Phillis and Limacher (130) iontophoretically applied bradykinin to spontaneously active cortical neurons including Betz cells. Ninety-one percent of all Betz cells and 76% of all other unidentified neurons responded to bradykinin. Guyenet and Aghajanian (131) found no response to bradykinin while recording from cells in the locus coeruleus. Cultured glioma and neuroblastoma X glioma hybrids were found to be hyperpolarized by bradykinin (132). By altering the content of specific ions in the culture dish and using specific ion channel blockers, the hyperpolarization to bradykinin was found to be due to increased potassium influx. In iontophoretic experiments, as well as with the injection of drugs directly into the brains of experimental animals, it is not possible to know the effective concentration of a drug at its site of action. Thus, these biological experiments are inadequate for demonstrating that brain tissue has a reasonable specificity and sensitivity to kinins. This shortcoming could be reduced by reversal of biological responsiveness by an appropriate

antagonist. Unfortunately, no suitable antagonist exists, and as a consequence the specificity of kinin action in brain cannot be assured.

Significance

The kallikrein-kinin system is a diverse and complex system whose function is not completely understood. Though several physiological mechanisms have been suggested to include kinins (15-23), none are considered to explicitly require the presence of these peptides. This is particularly true concerning kinin actions on CNS tissue. While an action of kinins in brain distinct from peripheral effectors has clearly been demonstrated (72, 118, 121), questions concerning the location, pathways, and mechanisms of action by kinins remain. For kinins to be identified as neurotransmitters or neuromodulators they should meet eight criteria (modified from 133):

1. They should be localized to the presumed releasing cell(s).
2. They should be synthesized or accumulated in the releasing cell(s) by a specific mechanism.
3. They should be released from the releasing cell(s) when the system is physiologically activated.
4. They should have access to specific receiving cells.
5. Specific recognition sites should be present on the receiving cell(s).
6. Exogenous administration of kinins to receiving cells should mimic the response produced by physiological activation of the system.
7. A system for inactivation of kinins should exist in the vicinity of the receptor.

8. Kinin antagonists should block the action of exogenous kinins and antagonize the physiologically activated system.

Within this dissertation will be presented evidence directed at helping to fulfill the fifth, sixth and seventh criteria in the list above. Data demonstrating specific recognition sites in brain tissue will be presented. The injection of kinins into the brain ventricles of conscious rats will be used to demonstrate the biological activity of the peptide, as well as help localize the biological activity to a specific region of the ventricular surface. Additionally, data showing the presence of kinin degrading activity in brain tissue and the inhibition of degradation by known protease inhibitors will be presented.

CHAPTER II
LOCALIZATION OF THE CENTRAL PRESSOR ACTION OF BRADYKININ TO THE THIRD VENTRICLE

Rationale

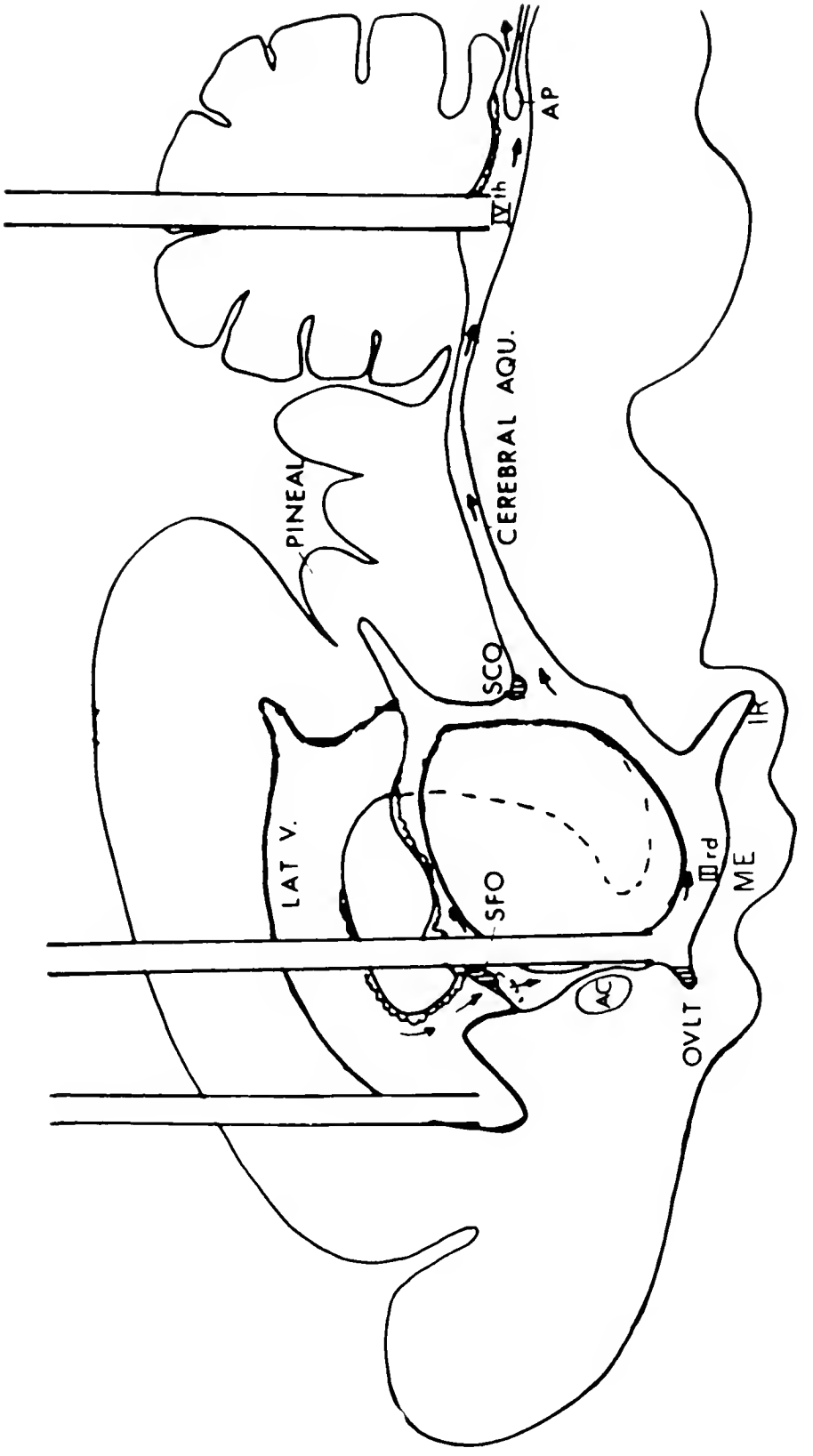
Bradykinin and kalladin (Lys-bradykinin), which are potent vasodilators in the peripheral circulation (134), have been found to elicit a pressor response when injected into the brains of rats (72, 118, 119). The mechanism of action of the pressor response may involve antidiuretic hormone (ADH). Baertschi and colleagues (135, 136) suggested the kinin-induced ADH release is the result of a central action of the peptide on the hindbrain. Hoffman and Schmid (118) showed that the antidiuretic response to central bradykinin appears to be at least partially responsible for the rise in systemic blood pressure. An attempt has been made to localize this effect. Using ivt injections of bradykinin into the lateral ventricle prior to and following selective electrolytic lesions, Correa and Graeff (137) concluded that the ventrolateral septal region was the locus for the kinin induced central pressor response. In the present report ventricular plugging (138-140) is used to determine the periventricular site or sites of action. Bradykinin injections combined with intraventricular plugs to restrict the flow of CSF were used to localize the pressor response. The data suggest that access to the ventral third ventricle is essential for the central kinin pressor response.

Methods

Under chloral hydrate anesthesia (400 mg/kg), female Sprague-Dawley retired breeders (Holtzman, 340-420g) were stereotaxically fitted with two intraventricular cannulae made from 23 gauge stainless steel tubing 14 mm in length. Each animal received a lateral ventricle cannula 1.0 mm posterior to bregma, 1.0 mm lateral from the midline and 5.0 mm deep from the dura. Each animal was also simultaneously implanted with either a third ventricle or fourth ventricle cannula of the same material (see Figure 3). Third ventricle cannulae were inserted 1.0 mm posterior to bregma, 1.0 mm lateral of the midline and 7.0 mm deep from the dura at an angle of 10° from the vertical. Coordinates of cannulae implanted into the fourth ventricle were 3.0 mm posterior from lambda, 0.0 mm lateral from the midline and 7.0 mm deep from the dura. Cannulae were anchored to the skull by jewelers screws embedded in dental acrylic cement. All rats were allowed three days recuperation before insertion of an indwelling catheter for measurement of blood pressure. Each silastic catheter was filled with heparinized sterile saline and inserted into the right femoral artery. After being secured by suture, the catheter was slipped underneath the skin and exteriorized on the back of the animal. A closed-end stylus plugged the free end of the tubing to maintain patency. This scheme allowed recording of blood pressure while the animal was conscious and unrestrained. Testing was performed on the day following catheterization.

Cream plugs injected into specific ventricular locations through properly oriented cannulae deny drug access to those locations (139, 140). The plug consisted of cold cream (Nivea) and was injected by

FIGURE 3: Regions of ventricular cannulae placement. Two cannulae were implanted in each animal: one cannula in the lateral ventricle for the injection of drugs, and one cannula in either the anterior third ventricle or the fourth ventricle for the injection of cream plugs. OVLT, organum vasculosum of the lamina terminalis; SFO, subfornical organ; Lat. V., lateral ventricle; AP, area postrema; ME, median eminence; IR, infudibular recess; AC, anterior commissure.


AP
IVth
CEREBRAL AQU.
PINEAL
SCO
LAT V.
SFO
IIIrd
ME
IR
AC
OVLT

filling a short section of PE 50 tubing calibrated to a known volume of 10 µl (third ventricle plugs) or 6 µl (fourth ventricle plugs). The filled polyethylene tube was then fitted to a saline-filled 1 ml syringe at one end and to the appropriate cannula via a short section of 23 gauge tubing at the other end.

For plug injections animals were lightly anesthetized with ether. The saline-filled syringe was slowly compressed so that injection of the cream could be observed as it moved through the short length of calibrated tubing. Each injection took approximately two minutes. Two hours of recovery were allowed before testing. Plug placement had no outward effect on the animals' behavior. Each animal was alert and responsive. Mean basal blood pressure was unchanged following plug injection (Third ventricle plugs: 106 ± 3 mm Hg before plug vs 104 ± 3 mm Hg following plug; Fourth ventricle plugs: 96 ± 4 mm Hg before plug vs 96 ± 6 mm Hg following plug).

One hundred nanograms angiotensin II (Ciba-Geigy) or 5 µg bradykinin triacetate (Sigma) were injected in 1 µl volumes through 30 gauge injectors attached via polyethylene tubing to a Hamilton syringe. The injectors were constructed so that when inserted their tips ended just beyond the tip of the lateral ventricle cannula.

At the end of each experiment the rat was injected with 1 - 3 µl of black ink and after several minutes was decapitated. The brain was excised and immersed in 40% formaldehyde. On subsequent days brains were sectioned parasagittally on a freezing microtome. The location and patency of each plug were recorded.

All data presented are paired values given as mean ± standard error. Results were evaluated by paired Student t-tests.

The study was performed in two parts. First, pressor responses to ivt angiotensin II and bradykinin were recorded. In the case of angiotensin II drinking was also measured. Following these initial tests a ventral third ventricle plug was inserted as described above. Following a two-hour recovery period each animal was tested a second time with each drug. One hour was permitted between drug injections and the order of both pre- and post-plug injections was randomized.

The second experiment consisted of intravenous injections of bradykinin before and after cream plug insertion into the fourth ventricle. The same procedure was followed as described above.

Results

Third Ventricle Plugs

Previously published results using cream plugs (140) suggested that the site of angiotensin II action was the ventral anterior third ventricle. In an attempt to confirm this finding and to pharmacologically verify the location of the cream plugs, five rats were challenged with 100 ng angiotensin II injected into the lateral ventricle before and after plugging. Figure 4 and Figure 5b show the pressor and drinking responses recorded. Ventricular plugs significantly depressed the pressor response (22 ± 3 mm Hg pre-plug vs 4 ± 2 post-plug, $p < .005$, $N = 5$) and drinking response (3.7 ± 0.6 ml pre-plug vs. 0.9 ± 0.6 ml post-plug, $p < .005$, $N = 5$).

Pressor responses to 5 µg bradykinin injected into the left ventricle following third ventricle plugging were significantly lower

FIGURE 4: Pressor and drinking responses to angiotensin II injections before and after anterior third ventricle obstruction. Left: change in the mean arterial blood pressure to 100 ng angiotensin II ivt before and after plug insertion.

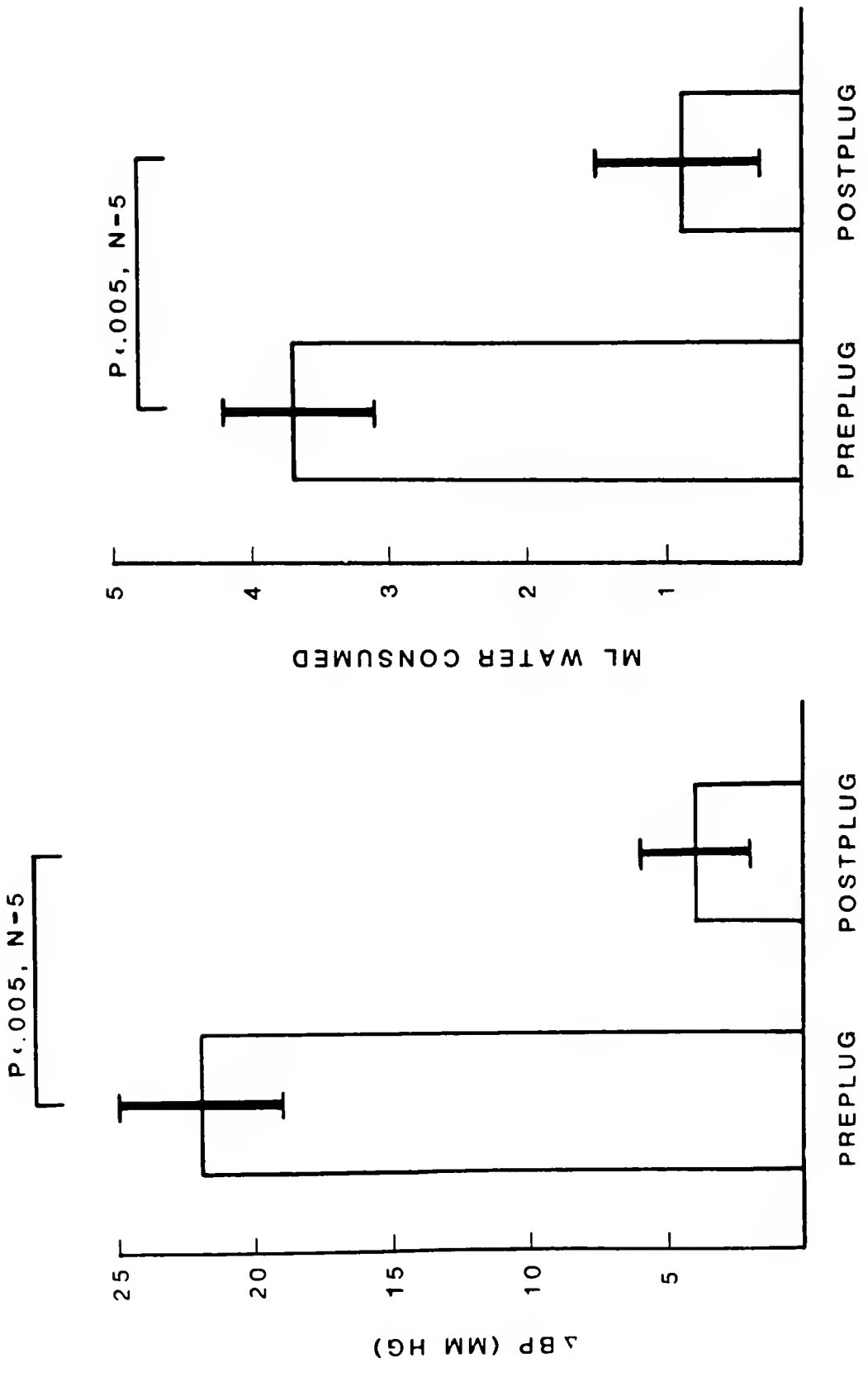

P<.005, N=5
5
4
3
2
1
ML WATER CONSUMED
PREPLUG
POSTPLUG
P<.005, N=5
25
20
15
10
5
Δ BP (MM HG)
PREPLUG
POSTPLUG

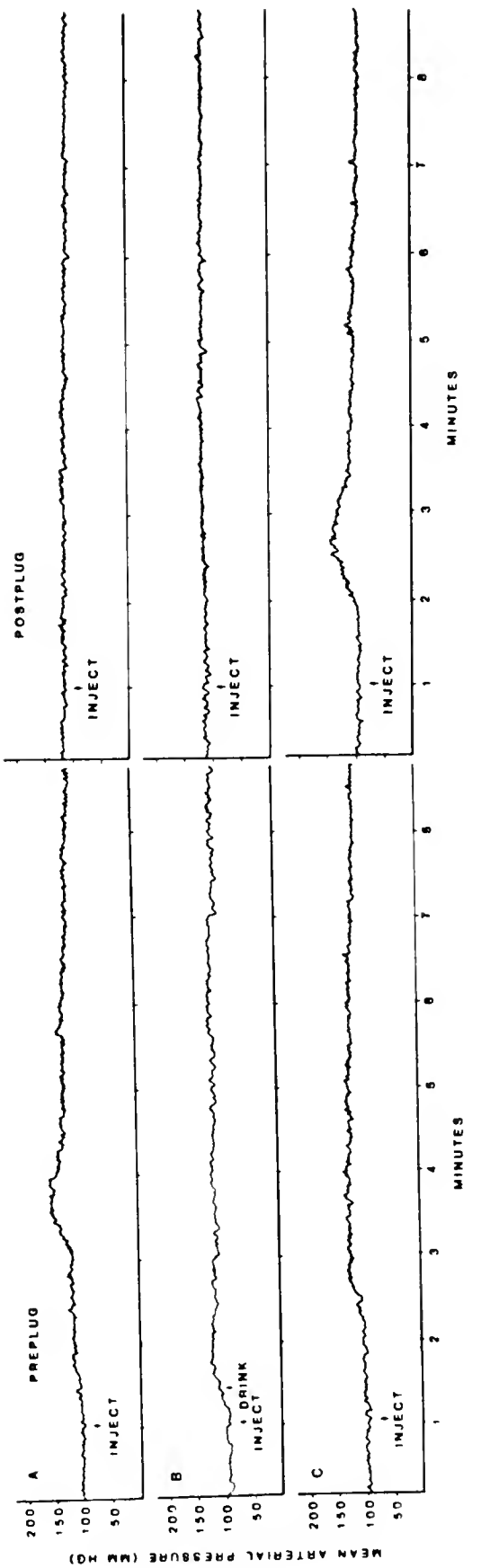
FIGURE 5: Examples of mean blood pressure recordings in three animals injected with

(A) 5 µg bradykinin ivt before and after anterior third ventricle plugging;

(B) 100 ng angiotensin II ivt before and after anterior third ventricle plugging or;

(C) 5 µg bradykinin ivt before and after fourth ventricle plugging.

Anterior third ventricle plugs were effective in blocking pressor responses to ivt injected drugs, whereas fourth ventricle plugs were not.


A
PREPLUG
INJECT
POSTPLUG
INJECT
B
DRINK
INJECT
INJECT
C
INJECT
INJECT
200
150
100
50
MEAN ARTERIAL PRESSURE (MM HG)
1
2
3
4
5
6
7
8
MINUTES
MINUTES

than before insertion of the plugs (Figure 5a and Figure 6, left panel, 27 $\pm$ 5 mm Hg pre-plug vs 5 $\pm$ 5 mm Hg post-plug, $p < .025$, $N = 7$).

Histology of rat brains with third ventricle plugs confirmed the correct position of all plugs. In every animal cream plugs obstructed access of CSF to the anterior ventral third ventricle. In five of seven cases plugs prevented access of ink injected into the lateral ventricle beyond the foramen of Munro. In the two instances when ink passed the foramen and traversed the third ventricle, the plugs securely blocked access to all sites on the anterior ventral and posterior ventral third ventricle. An example of the third ventricle plug is shown in Figure 7.

Fourth Ventricle Plugs

Pressor responses to 5 µg bradykinin before and after fourth ventricle plugging are illustrated in Figure 5c and Figure 6 (right panel). Lateral ventricular injections elevated mean arterial blood pressure 27 $\pm$ 8 mm Hg before plug insertion and 35 $\pm$ 9 mm Hg after plug insertion ($p > .05$, $N = 5$). Histological analysis following testing confirmed plug localization within the fourth ventricle. Figure 8 demonstrates a typical fourth ventricle plug.

Discussion

The major finding of this study is that the central pressor response elicited by injected bradykinin into the lateral ventricle is localized in or requires access to the ventral portion of the third ventricle. CSF flows from the lateral ventricle to the third and fourth ventricles and out the arachnoid villi (141). Cream plugs blocking drug access from the lateral to the third ventricle blocked the pressor response. Correspondingly, cream plugs which blocked drug access to the

FIGURE 6: Change in mean arterial pressure to 5 µg bradykinin injected through lateral ventricle cannulae prior and subsequent to third ventricle (left) or fourth ventricle (right) plugging. Third ventricle plugs, but not fourth ventricle plugs, significantly attenuated the pressor response to ivt bradykinin.

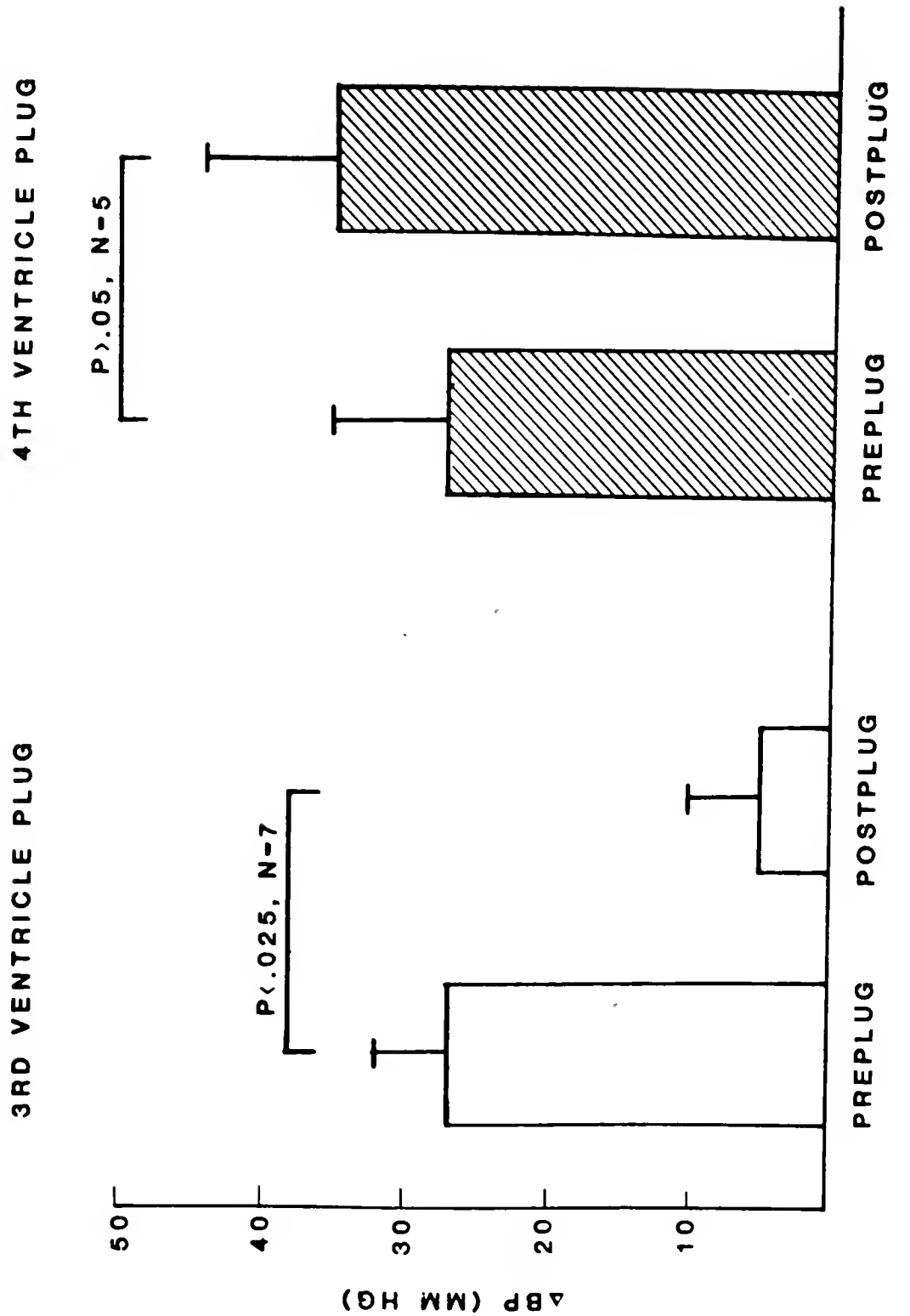

3RD VENTRICLE PLUG
4TH VENTRICLE PLUG
P<.025, N-7
P>.05, N-5
ΔBP (MM HG)
50
40
30
20
10
PREPLUG
POSTPLUG
PREPLUG
POSTPLUG

FIGURE 7: Plugging of the anterior ventral third ventricle area with a cream plug. Black dye injected through a third ventricular cannula (small arrows), after plugging, indicates the spread of injectate. The white plugged area (large black arrows) shows no mixing of black ink and this indicates a complete block of the area to diffusion within the ventricle. There was diffusion to the dorsal third ventricle (open arrow). With such blockade bradykinin was without a central effect.

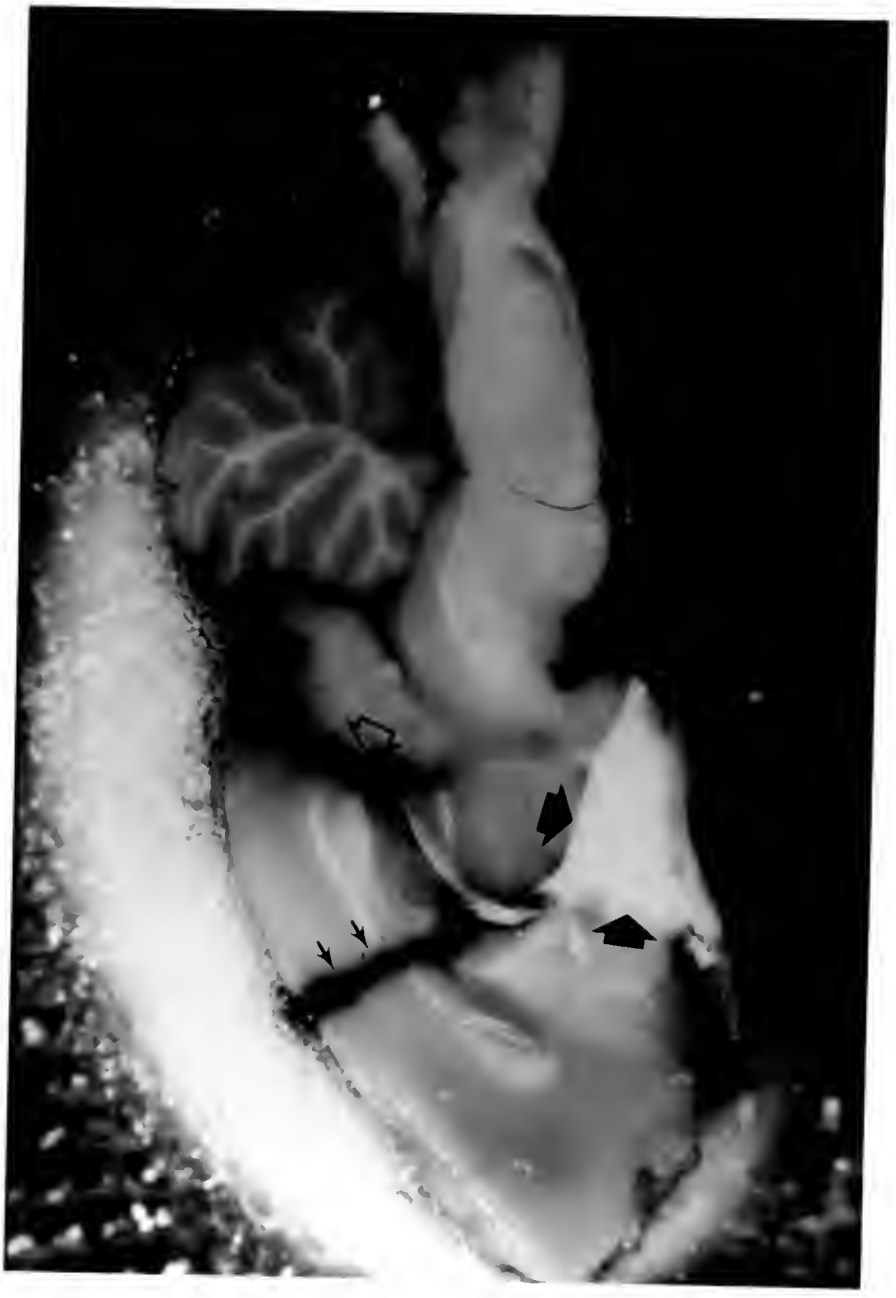
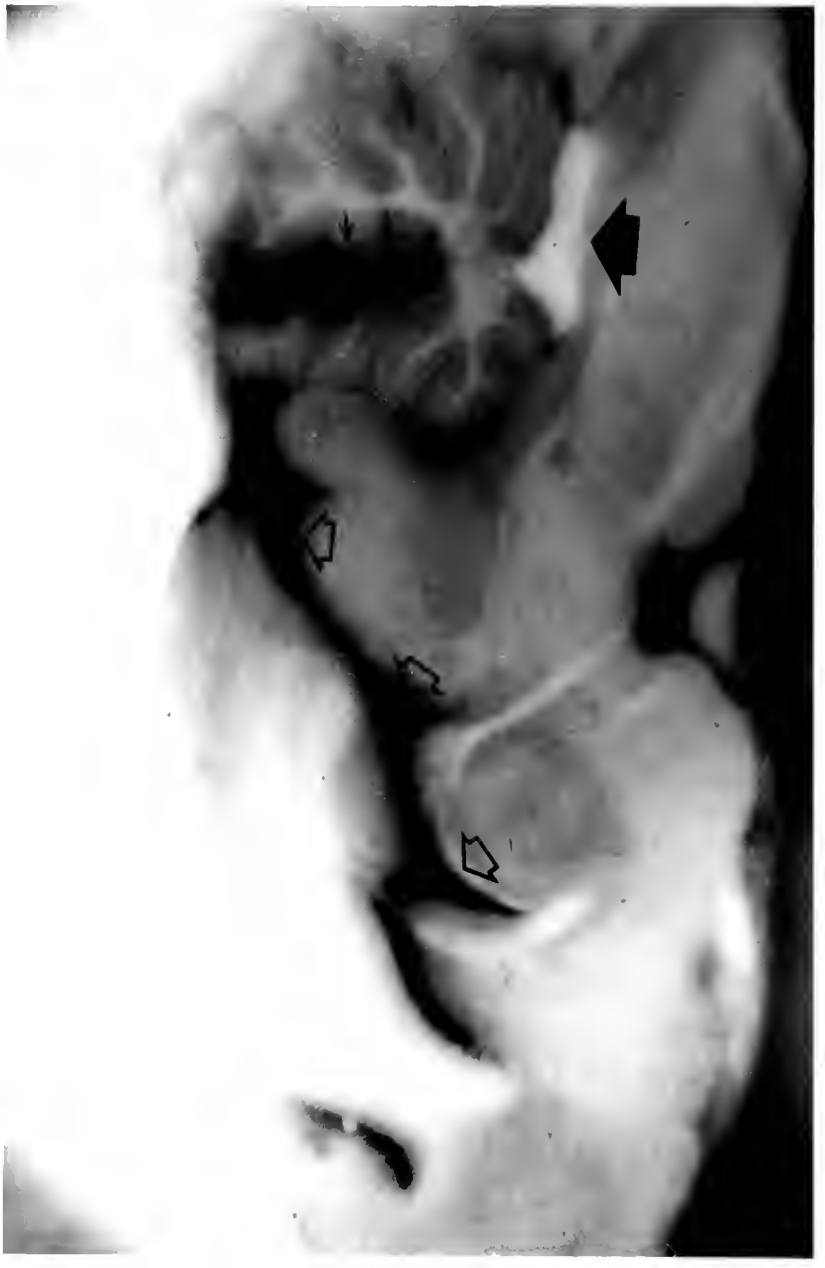


FIGURE 8: Plugging of the fourth ventricle (solid arrow). Black dye has been injected after plugging into the lateral ventricles. The open arrows indicate areas where the dye has spread but no dye has passed through the fourth ventricle plug. Small arrows indicate the projectory of the plugging cannula. Blockade of the fourth ventricle did not abolish the pressor effect of intraventricular bradykinin but potentiated it.



fourth ventricle, but not to the cerebral aqueduct or third ventricle, did not block the bradykinin pressor response. In two instances where cream plugs denied drug access to the ventral third ventricle, but allowed passage to the dorsal third ventricle, cerebral aqueduct and fourth ventricle structures, the bradykinin pressor response was still absent. The patency of third ventricle plugs was confirmed by ink injection and histological section and also by cream plug obliteration of the pressor and drinking responses to angiotensin II injected into the lateral ventricles.

The results of the cream plug on intravenous angiotensin II action support previous findings using this technique (140). That report concluded that the site of action of angiotensin II for the pressor and drinking responses was the anterior ventral third ventricle.

Correa and Graeff (137) suggested from lesion experiments that the site of action of the central kinin pressor response is the ventral lateral septal region. However, their study does not rule out two alternative explanations of their results: (1) the possibility that they lesioned a portion of the pathway mediating the central kinin pressor response and not the receptor site for bradykinin; and (2) the site of the lesion was so close to the foramen of Munro that post-operative swelling effectively blocked passage of the drug to distal structures.

Baertschi and colleagues (135, 136) concluded from responses to bradykinin systemically injected so as to pass through the vertebral arteries that the peptide had a rhombencephalic locus of action on the neurohypophysis. ADH release has been reported as a consequence of

bradykinin action on the brain when injected into the blood (142) or intracerebroventricularly (118). Hoffman and Schmid (118) report that the release of ADH is partially responsible for the central kinin pressor response. Presumably for bradykinin to penetrate to the brain it would require access to a circumventricular organ where there is no blood brain barrier. In the hindbrain only the area postrema is known to lack a blood brain barrier. Results from fourth ventricle plugs can be used to argue against this site of action. The possibility that systemically injected bradykinin acts on the blood side of the neural tissue cannot be excluded from the results of Baertschi et al. (135, 136), nor, however, can the possiblity that bradykinin in their study traversed the vertebral and basilar arteries to reach the posterior cerebral artery. The posterior cerebral artery supplies the caudal diencephalon among other regions (143). Results reported here are consistent with this possibility.

Correa et al. (104) recently reported kinin-like immunoreactivity in rat brain. Immunofluorescent cell bodies were observed only in the medial basal hypothalamus--a ventral third ventricle structure. Evidence exists for an endogenous brain-kinin system (107, 112); however, specific kinin receptors have yet to be identified in brain tissue.

That the site of action of bradykinin and angiotensin II can be localized to the ventral third ventricle is interesting in light of previous comparisons between the central actions of angiotensin II and bradykinin (118, 125).

CHAPTER III
INVESTIGATING THE CENTRAL ACTION OF BRADYKININ: POSSIBLE INTERACTIONS WITH ANGIOTENSIN II

Rationale

Recent advances in research on central actions of peptides suggest that central mechanisms of cardiovascular control may be mediated or modulated by brain peptides. In this study a peptide-peptide interaction on blood pressure was investigated by focusing on the cardiovascular responses of angiotensin II and bradykinin. When these peptides are injected into the brain ventricles they both cause an increase in mean arterial blood pressure (72, 117, 118, 144). The peripheral action of these peptides, however, is quite different. Intravenous angiotensin II increases blood pressure but intravenous bradykinin produces vasodilation. Bradykinin is degraded by the enzyme kininase II, the same enzyme that has the role of converting angiotensin I to angiotensin II (145). Thus, the two peptides would seem to have an interaction which could be tested by the use of converting enzyme inhibitors and angiotensin II antagonists. In addition, angiotensin II has more prolonged effects in the brain when prostaglandin synthesis is inhibited (146), whereas the central pressor effect of bradykinin is attenuated by the prostaglandin synthesis inhibitor indomethacin (121). It has been reported that prostaglandin E_1 and E_2 will inhibit angiotensin II-induced drinking in the rat (147).

To test if there is a central interaction between two neuropeptides with cardiovascular effects we have studied selected doses of bradykinin that are known to be effective and reliable in inducing pressor responses when injected into the brain. We have found an interaction between angiotensin II and bradykinin on pressor responses to ivt injections.

Methods

Surgery

All experiments were performed on conscious unrestrained male Sprague-Dawley rats. Each rat was surgically prepared under chloral hydrate anesthesia (400 mg/kg) with 23 gauge, 14 mm long stainless steel cannula at least 3 days before testing. Each cannula was placed in the lateral ventricle: 0.5 or 1.0 mm posterior, 1.0 mm lateral, 5.0 mm deep (from dura) with respect to bregma (flat skull). For blood pressure recording a chronic indwelling catheter was inserted into the femoral artery and vein under ether anesthesia. Each catheter was slipped underneath the skin and exteriorized on the back of the animal.

Equipment and Solutions

Blood pressure was continuously measured with a Statham P23Gb pressure transducer and heart rate was measured by a cardiotachometer. Changes in blood pressure and heart rate were recorded on a Beckman R411 Dynograph Recorder. When a central drug pretreatment was called for, the infusion was given through the same intracranial cannula used for kinin injections. Drug infusions were administered in 1-5 µl volumes. Bolus intracranial injections were given by hand with a 10 µl or 25 µl Hamilton syringe.

Solutions containing angiotensin II (Ciba), bradykinin, Lys-bradykinin, Des-Arg^9-bradykinin, Tyr-bradykinin (Penninsula and Bachem), (Sigma), saralasin acetate (Calbiochem-Behring), or SQ 14,225 (Captopril, D-3-mercapto-2-methylpropanoyl-L-proline, Squibb) were prepared in 0.9% sterile saline at neutral pH. Indomethacin (Upjohn) was dissolved in 1.0 M NaOH and titrated to pH 7.4 with HCl or dissolved by stirring at 40° C in 0.9% saline buffered to pH 7.4 with 23 mM $NaHCO_3$. The doses of bradykinin, indomethacin and angiotensin II were chosen from dose response curves published previously (118, 144, 148).

Procedures

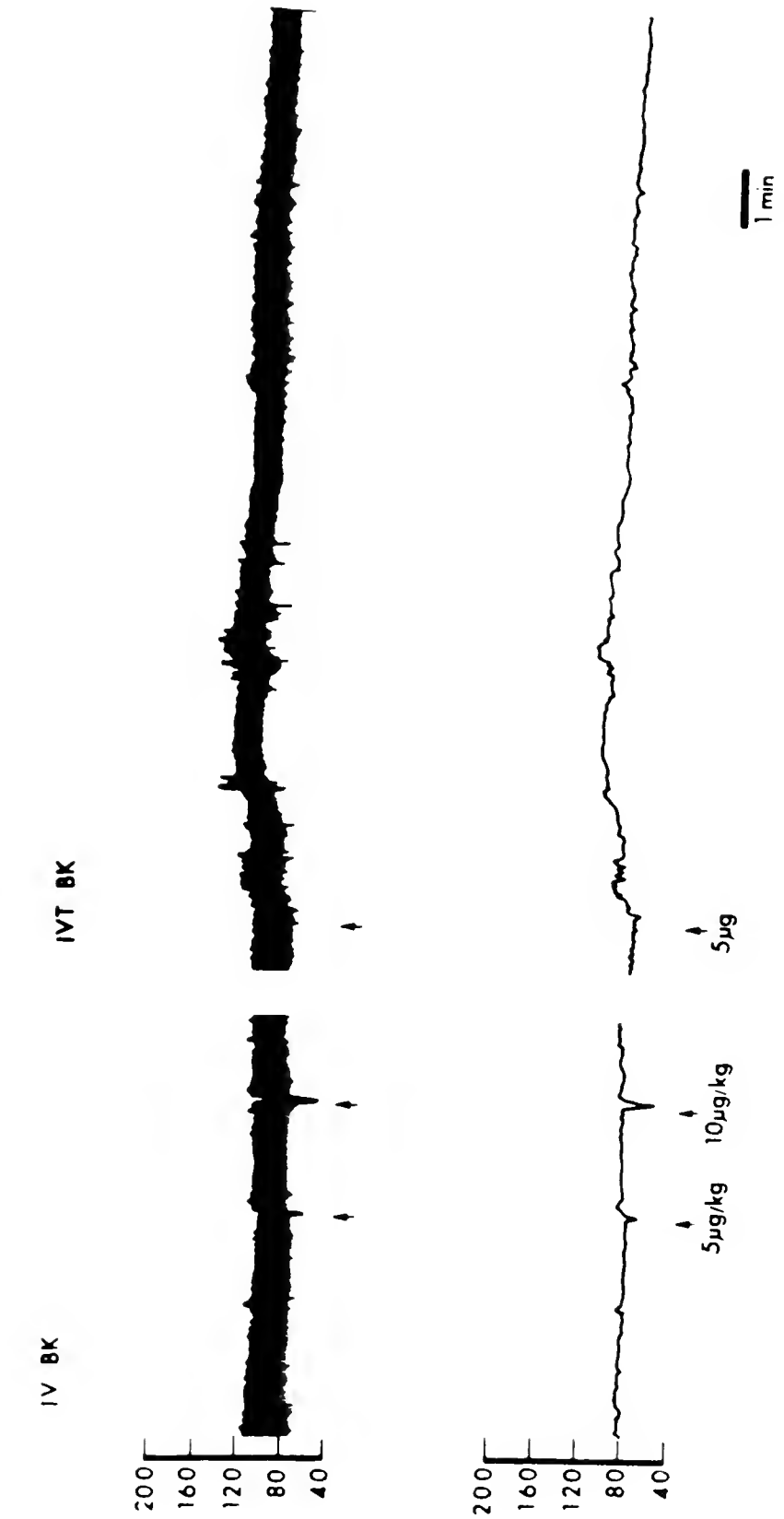
Separate tests were carried out in different groups of rats. Blood pressure responses to kinins and angiotensin II were conducted in separate tests. In all tests the order of testing was randomized. In tests conducted where a change in responsiveness followed indomethacin treatment, at least 2 hours were allowed between tests. All other tests were separated by 45 minutes or more. All values are reported as mean ± standard error. Comparisons were made with a paired or unpaired Student t-test, or analysis of variance and Newman-Keuls test, where appropriate.

Results

Figure 9 demonstrates the difference in blood pressure changes to intravenous and ivt injections of bradykinin. The intravenous routes produced a brief depressor and the ivt route of injection produced a long-lasting pressor effect.

Pressor responses were of short latency for ivt injections of 200 ng angiotensin II (20 ± 3 mm Hg, 25 ± 4 sec) and 5 µg bradykinin

FIGURE 9: Example of the peripheral and central effects of bradykinin in a rat. Intravenous bradykinin induces a depressor effect, but in the same animal intraventricular bradykinin produces a pressor effect. Upper trace: pulse pressure. Lower trace: mean pressure recorded from femoral artery of a conscious unrestrained rat.


IV BK
IVT BK
200
160
120
80
40
5µg/kg
10µg/kg
5µg
1 min

(18 ± 5 mm Hg, 37 ± 10 sec). Duration of the pressor response, i.e., the time from beginning of response until return to baseline, was 15 ± 4 min for angiotensin II and 18 ± 6 min for bradykinin. Treatment with 10 μg saralasin ivt 3-5 minutes prior to injection of 200 ng angiotensin II significantly reduced the pressor response (Figure 10, $P < .005$ measured by a paired t-test) and associated bradycardia (Figure 11, $P < .05$ measured by a paired t-test). The same dose of saralasin ivt, however, potentiated the pressor response to 5 μg bradykinin ivt (Figure 10, $P < .01$ measured by a paired t-test) with no significant effect on the associated tachycardia (Figure 11). Saralasin alone at the dose used had no effect on basal heart rate or blood pressure.

Pretreatment with 2 μg of the angiotensin converting enzyme inhibitor Captopril potentiated 1 μg ivt bradykinin but when given 5 minutes prior to 5 μg bradykinin ivt a drop in pressure resulted (Figure 12). Captopril alone did not affect blood pressure. The Captopril solution was acidic (pH 3.5) and therefore the vehicle (1 μl) with pH 3.5 was tested alone. No effect on blood pressure or drinking was observed.

The pressor and heart rate responses to 5 μg ivt bradykinin were also attenuated by 10 μg ivt indomethacin administered 3-5 minutes before injection of the peptide (Figure 13). Ivt injection of indomethacin alone did not alter blood pressure or heart rate.

The central pressor response to bradykinin was compared with Lys-bradykinin and other kinin analogs (Figure 14). Bradykinin, Lys-bradykinin and Tyr-bradykinin were all capable of elevating blood pressure above saline control injections. Des-Arg^9-bradykinin, however, did not

FIGURE 10: The change in blood pressure (BP) from pretreatment levels after angiotensin II (Ang II), saralasin (P113) combined with Ang II, bradykinin (BK) and saralasin with BK were given ivt. N = 5 for all experiments. Data are given as mean ± S.E.

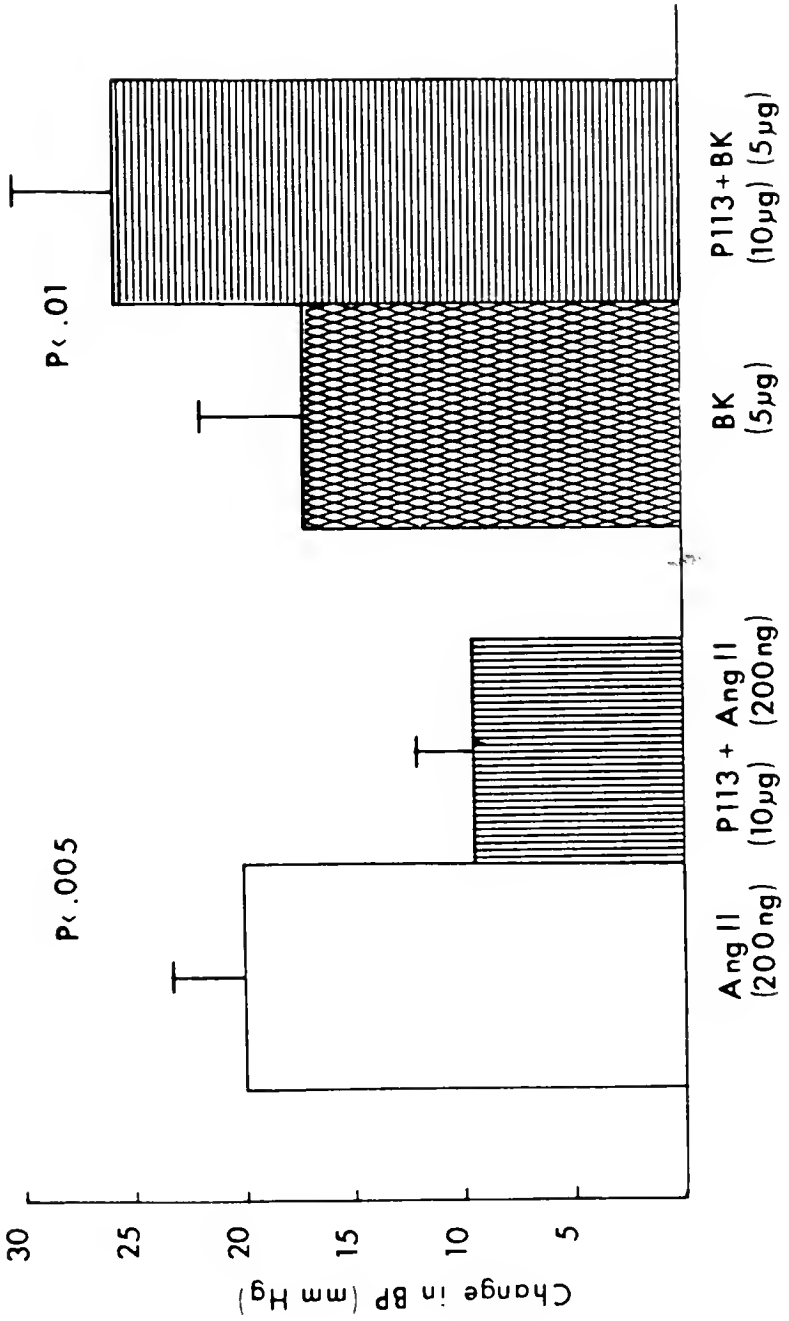

Change in BP (mm Hg)
30
25
20
15
10
5
P< .005
P< .01
Ang II (200 ng)
P113 + Ang II (10µg) (200 ng)
BK (5µg)
P113+BK (10µg) (5µg)

FIGURE 11: The change in heart rate (HR) in beats per minute (bpm) following treatment with angiotensin II, saralasin, bradykinin and combinations of saralasin and the two peptides. All doses were given ivt. N = 5 for all experiments. Data are given as mean ± S.E.

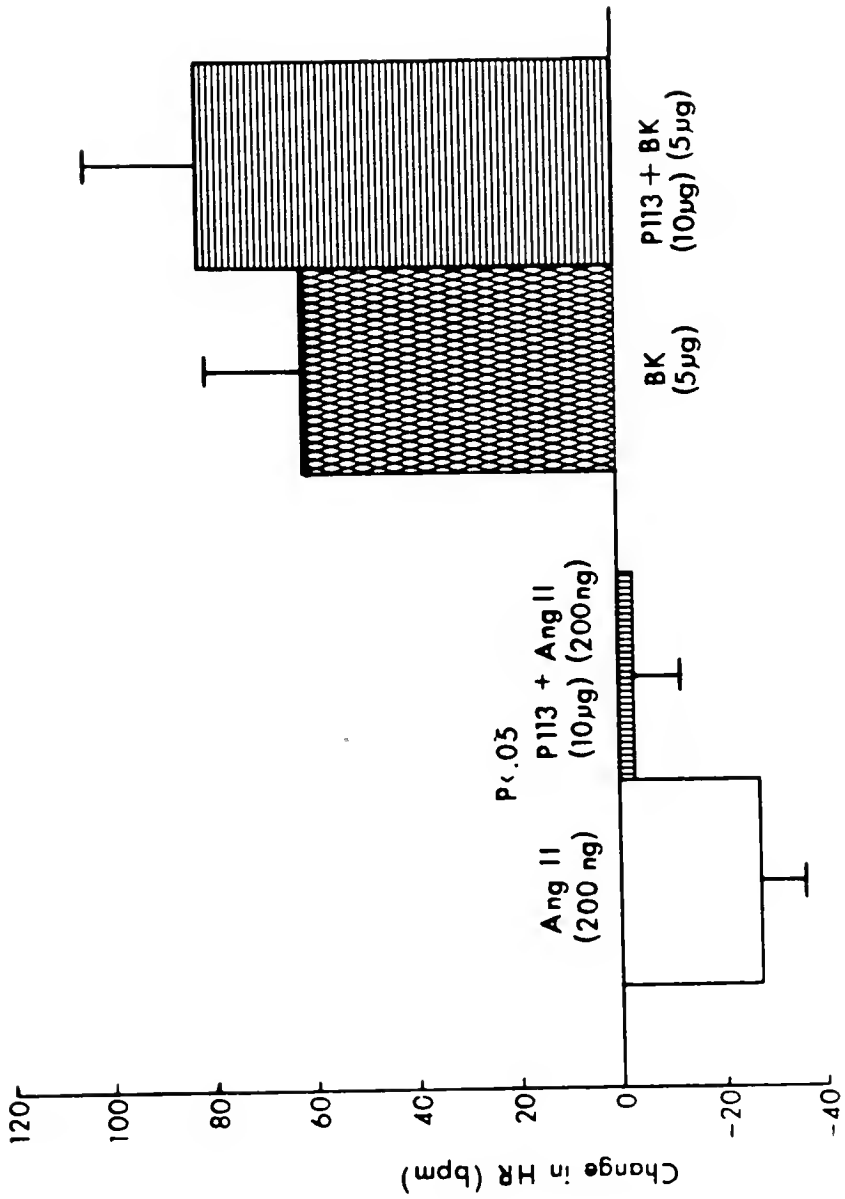

Change in HR (bpm)
120
100
80
60
40
20
0
-20
-40
Ang II (200 ng)
P<.05
P113 + Ang II (10µg) (200ng)
BK (5µg)
P113 + BK (10µg) (5µg)

FIGURE 12: Change in blood pressure to different doses of bradykinin ivt with (open circles) and without (closed circles) Captopril ivt. Captopril alone demonstrated no effect on blood pressure. BK = bradykinin; data are given as mean ± S.E.; * $P < .001$.

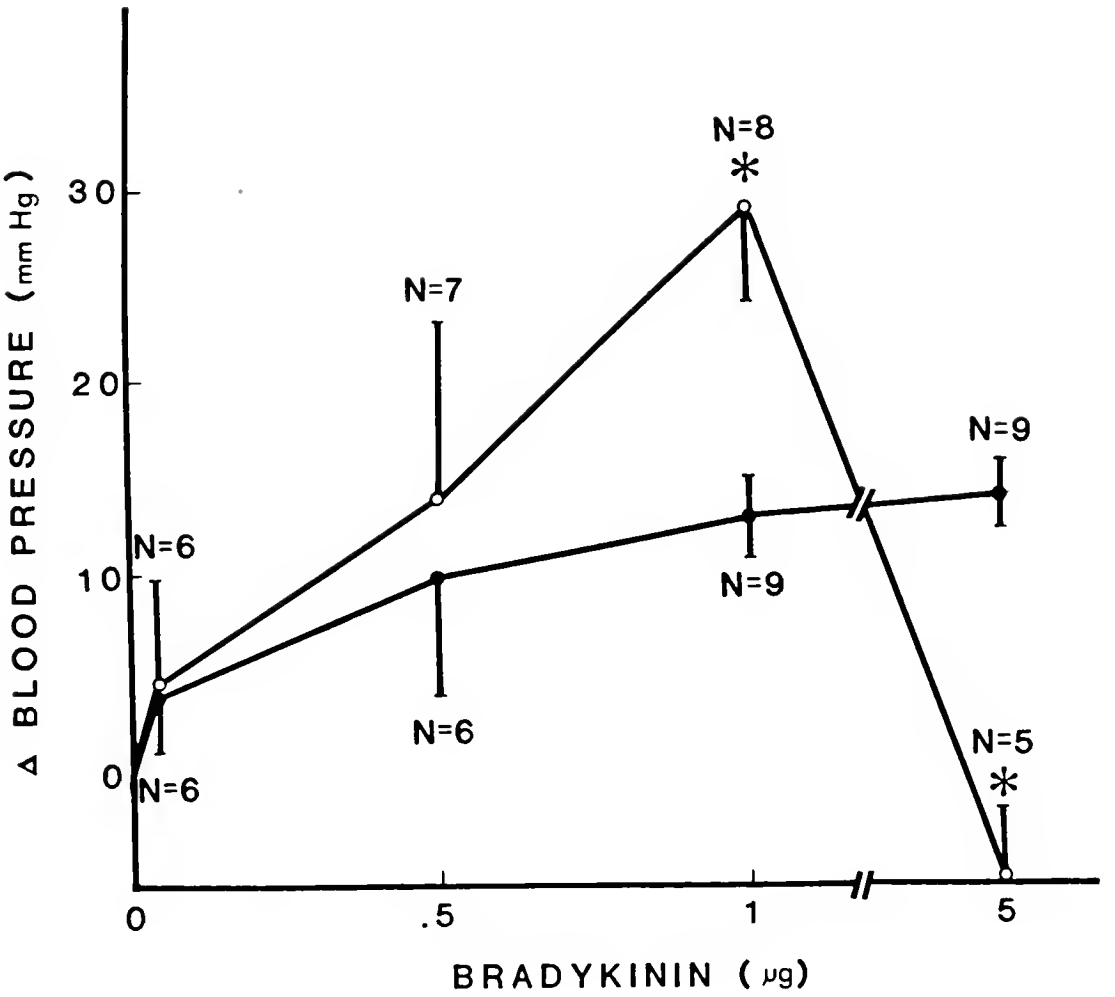

Δ BLOOD PRESSURE (mm Hg)
30
20
10
0
N=8
*
N=7
N=9
N=6
N=9
N=6
N=6
N=5
*
0
.5
1
5
BRADYKININ (μg)

FIGURE 13: Attenuation of the blood pressure and heart rate response to bradykinin (BK) ivt by indomethacin (IM) pretreatment ivt.

Left: Change in blood pressure from pretreatment level.
Right: Change in heart rate from pretreatment level.

Indomethacin alone demonstrated no effect on blood pressure or heart rate. Data are given as mean ± S.E.

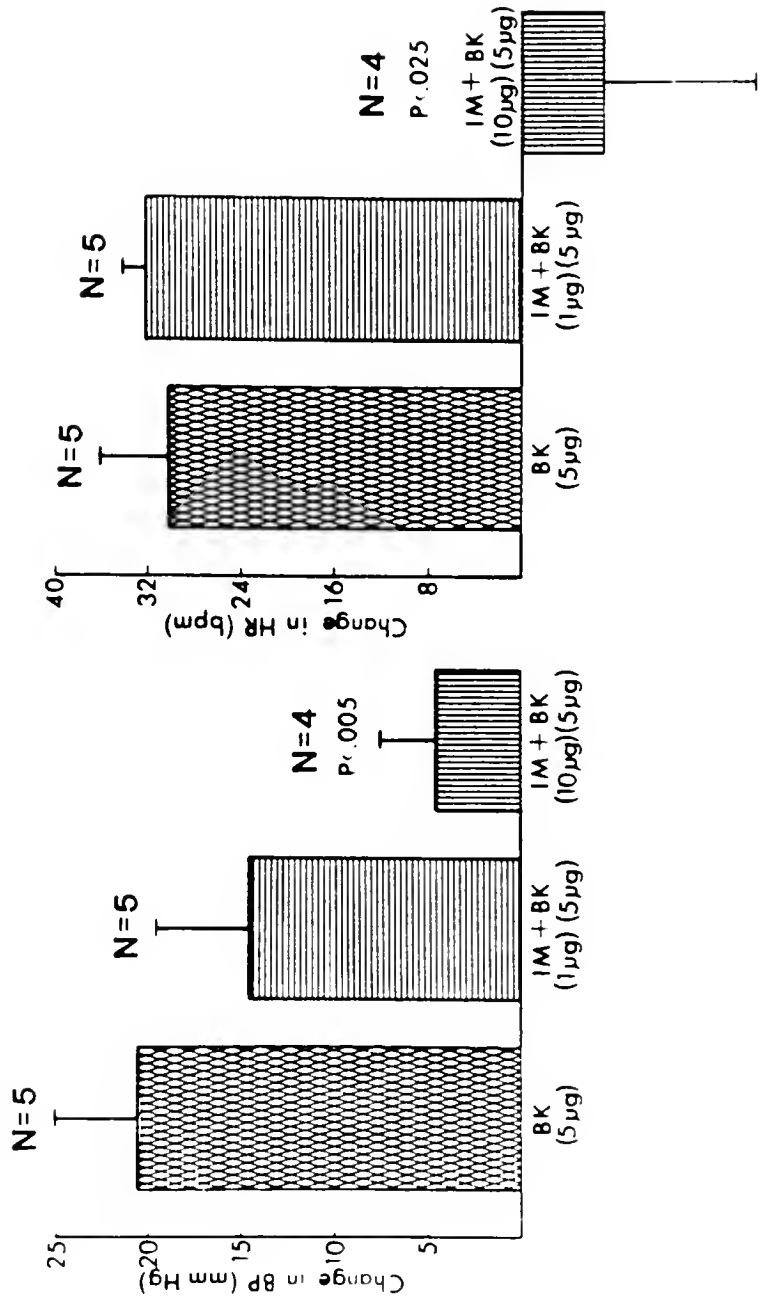
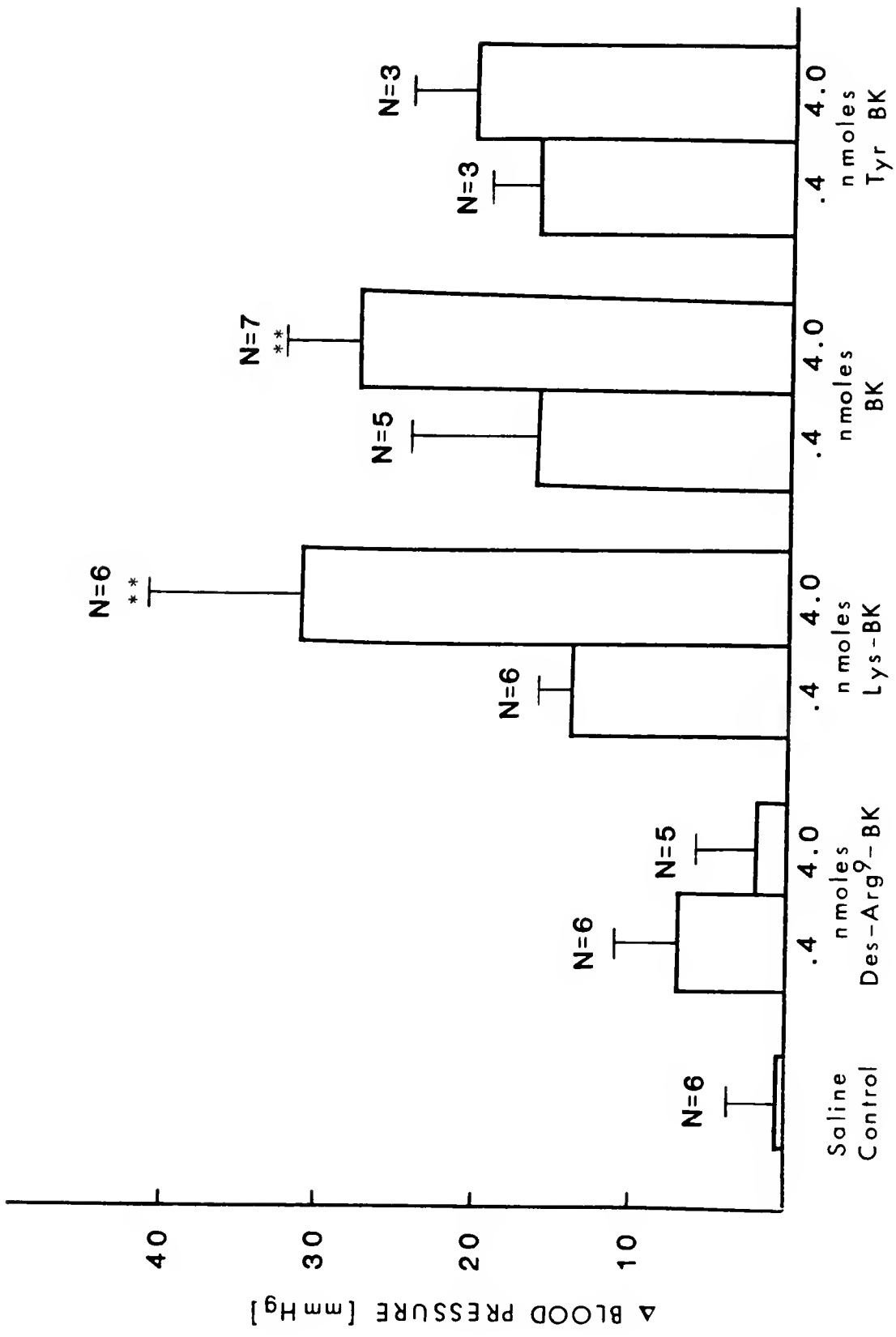

Change in BP (mm Hg)
25
20
15
10
5
N=5
BK (5μg)
N=5
IM + BK (1μg) (5μg)
N=4
P<.005
IM + BK (10μg)(5μg)
Change in HR (bpm)
40
32
24
16
8
N=5
BK (5μg)
N=5
IM + BK (1μg) (5μg)
N=4
P<.025
IM + BK (10μg) (5μg)

FIGURE 14: Change in blood pressure to control saline injections, or two doses of Des-Arg^9-bradykinin (Des-Arg^9-BK), kalladin (Lys-BK), bradykinin (BK) or Tyr-bradykinin (Tyr-BK). Significance was tested for by analysis of variance and Newman-Keuls test. Data are given as mean ± S.E.; ** $P < .01$ when compared to control or to 4.0 nmoles of Des-Arg^9-BK.


Δ BLOOD PRESSURE [mmHg]
40
30
20
10
Saline Control
N=6
Des-Arg9-BK
N=6
N=5
Lys-BK
N=6
N=6
**
BK
N=5
N=7
**
Tyr BK
N=3
N=3
.4
4.0
nmoles

elicit pressor responses at the doses tested which were significantly different from control injections.

Discussion

Angiotensin II has an action in the brain which elevates blood pressure (144, 149). Increases in arterial blood pressure following central administration of the nonapeptide bradykinin have also been reported (72, 118, 119). Additionally, Yang et al. (150) recognized that angiotensin converting enzyme and kininase II were one and the same. Given these facts we hypothesized that angiotensin II and bradykinin shared some relationship with respect to their pressor actions in the brain. We tested whether the peptides were in series, i.e., bradykinin causing the release of angiotensin II which, in turn, elicits the pressor response. If this were true it would be predicted that the pressor effect of central bradykinin could be abolished by the angiotensin II competitive antagonist saralasin. The two doses were selected to produce nearly equivalent rises in blood pressure on their own (20 ± 3 mm Hg for angiotensin II vs 18 ± 5 mm Hg for bradykinin). However, saralasin potentiated the pressor responses to bradykinin while attenuating the response to angiotensin II (Figure 10). The experiments suggest that the two peptides do not act in series and that angiotensin II inhibits the pressor action of bradykinin.

The possibility of saralasin inhibition of converting enzyme to produce greater amounts of bradykinin was considered. Chiu et al. (151) found a reduction in the conversion of angiotensin I to angiotensin II by porcine lung converting enzyme in the presence of an equimolar

concentration of saralasin. However, Fitz and co-workers (152) found little if any inhibition of the enzyme extracted from human lung or porcine plasma by saralasin. Further, converting enzyme/kininase II has a 10- to 100-fold greater affinity for bradykinin than for angiotensin I (88). Therefore, since the molarities of bradykinin and saralasin administered in this study were similar, it is unlikely that saralasin would have been a better competitor for kininase II than bradykinin.

If angiotensin II is inhibiting the bradykinin pressor response as the saralasin data indicate, then inhibition of angiotensin II formation should also enhance the bradykinin response. Figure 12 demonstrates potentiation of the pressor response to bradykinin during converting enzyme inhibition by Captopril. A similar result has recently been reported by Unger et al. (145). Their interpretation was that more bradykinin was available. However, the potentiation also supports the notion of a decrease in the inhibitory action of angiotensin II on the central bradykinin pressor response. At the highest dose, 5 µg of bradykinin in the presence of 2 µg Captopril resulted in a depressor rather than a pressor response. The decrease in blood pressure can be explained by leakage of the higher dose into the circulation. Peripherally, bradykinin is a vasodilator and peripheral vasodilation may override the central pressor response. Thus, Captopril may not only inhibit degradation of central bradykinin, but may also prevent its central inhibition by angiotensin II.

Injection of 50 ng angiotensin II with 5 µg bradykinin into the brain ventricles of conscious rats does not result in a pressor response significantly different from the response observed when either of the

two peptides is injected alone (153). It might be anticipated that the addition of two separate central pressor substances would have an additive effect upon blood pressure. However, the lack of additivity with coinjection of angiotensin II and bradykinin could be interpreted as the result of angiotensin II inhibition of bradykinin pressor activity. Five micrograms bradykinin ivt elicits a maximal pressor response, and additivity of an effect would not be expected when the system is already maximally stimulated.

Bradykinin's centrally induced tachycardia (Figure 11, Figure 13) is difficult to interpret in light of other studies. Hoffman and Schmid (118) report a mild tachycardia in conscious rats with 1.0 µg doses of ivt bradykinin, but they observed a bradycardia with 5.0 µg ivt bradykinin. Conflicting with the results of Hoffman and Schmid (118) are other reports of tachycardia induced by ivt bradykinin injections in conscious rats (154) and rabbits (123). A tachycardia to ivt bradykinin is consistent with the tachycardia observed with ivt prostaglandin E_1 or E_2 (155, 148), potential mediators of the central kinin response (Figure 13).

A relationship between bradykinin and prostaglandins in the peripheral circulation is well demonstrated. Bradykinin induces the formation of prostaglandins in kidney (156) and isolated blood vessels (157). Prostaglandins also raise blood pressure when injected into the brain ventricles (149). Consequently, we looked for a relationship between the central action of bradykinin and prostaglandin synthesis. In fact, indomethacin did reverse the heart rate and attenuate the pressor effects of centrally administered bradykinin (Figure 13). This

confirms the results of Kondo et al. (121) and suggests that prostaglandin formation plays a role in bradykinin's central pressor effect.

These experiments provide evidence for an interaction between two peptides in the brain, angiotensin and bradykinin. Evidence exists for the presence of both angiotensin and bradykinin in the brain (107, 144). Both peptides have been demonstrated immunocytochemically in different brain regions (104, 158). Specific angiotensin receptors have been demonstrated in brain membranes (159) and neuronal cell culture (160) although bradykinin receptors in the brain have not yet been established.

Several analogues of bradykinin showed similar abilities to induce a pressor response when injected into the brain ventricles. While Lys-bradykinin demonstrated the most potent pressor response, it was not statistically different from either bradykinin or Tyr-bradykinin. That Des-Arg^9-bradykinin could not elevate blood pressure significantly more than control saline injections suggests that the central kinin pressor response is not mediated through receptors with affinity for this peptide. Such receptors, designated B_1 by Regoli, have only been identified in selected peripheral vasculature (14).

CHAPTER IV
DEGRADATION STUDIES WITH A RADIOACTIVE ANALOGUE OF BRADYKININ

Rationale

The biological activity of kinins in CNS tissue (72, 117-132) suggests the presence of specific kinin receptors in brain as mediators of these functions. Investigating the existence of kinin receptors in CNS tissue would require demonstrating the specific, saturable binding of a radioactive kinin molecule to a suspension of brain membranes, or using the ligand in an in vitro preparation of brain cells. However, technical considerations preclude direct application of standard radioligand binding techniques. Two criteria must be fulfilled prior to a search for specific, high affinity kinin receptors in CNS tissue: 1) choice of an appropriate radioactive ligand, and 2) inhibition of degradation of the ligand by brain proteases. The following describes the procedures used for meeting these criteria.

Methods

Iodination

Iodination of Tyr-bradykinin (Penninsula, Bachem) was accomplished by a modification of the dilute chloramine T method of DeMeyts (161). Twenty-five microliters of sodium phosphate buffer (pH 6.7) and 0.5 µg Tyr-bradykinin in 10 µl phosphate buffer were added to 0.5 mCi $Na^{125}I$ (Amersham) and 10 µl chloramine T (40 µg/ml) and incubated

for five minutes at 24° C to react all of the oxidizing reagent. This precludes the addition of reducing reagents such as sodium metabisulfite to quench the reaction. The reaction mixture was applied to a 5 ml Dowex 1 x 4, 200 mesh column (Sigma). Iodinated Tyr-bradykinin was eluted from the column with water. Twenty-drop fractions were collected.

Specific activity was determined by counting 10 µl aliquots of each fraction collected. Recovery was 95%. Thus (using a counting efficiency of 74%) the calculated number of µCi collected from the Dowex column was divided by 0.5 µg Tyr-bradykinin to yield the specific activity. Peak fractions from the Dowex column were pooled, diluted 50:50 with 100% ethanol and stored at -20° C.

Purity of the iodinated peptide was determined by HPLC. An aliquot of the pooled fractions from iodination was eluted on a methanol-ammonium acetate gradient. One milliliter fractions were collected at a flow rate of 1 ml/minute. The purity of the peptide was assessed by dividing the cpm in the kinin peak by the total cpm added. The location of the kinin peak was previously determined by addition of fresh, cold Tyr-bradykinin and observing an absorbance peak at 230 nM.

Degradation

Degradation experiments were carried out by incubation of ^{125}I-Tyr-bradykinin with either homogenates of whole brain or with cultured brain cells in the presence or absence of various inhibitors. Whole brain homogenate was prepared by homogenizing the brains of female Sprague-Dawley rats in 10 volumes of 25 mM potassium phosphate buffer (pH 7.2) with a polytron at maximum speed for 30 seconds followed by

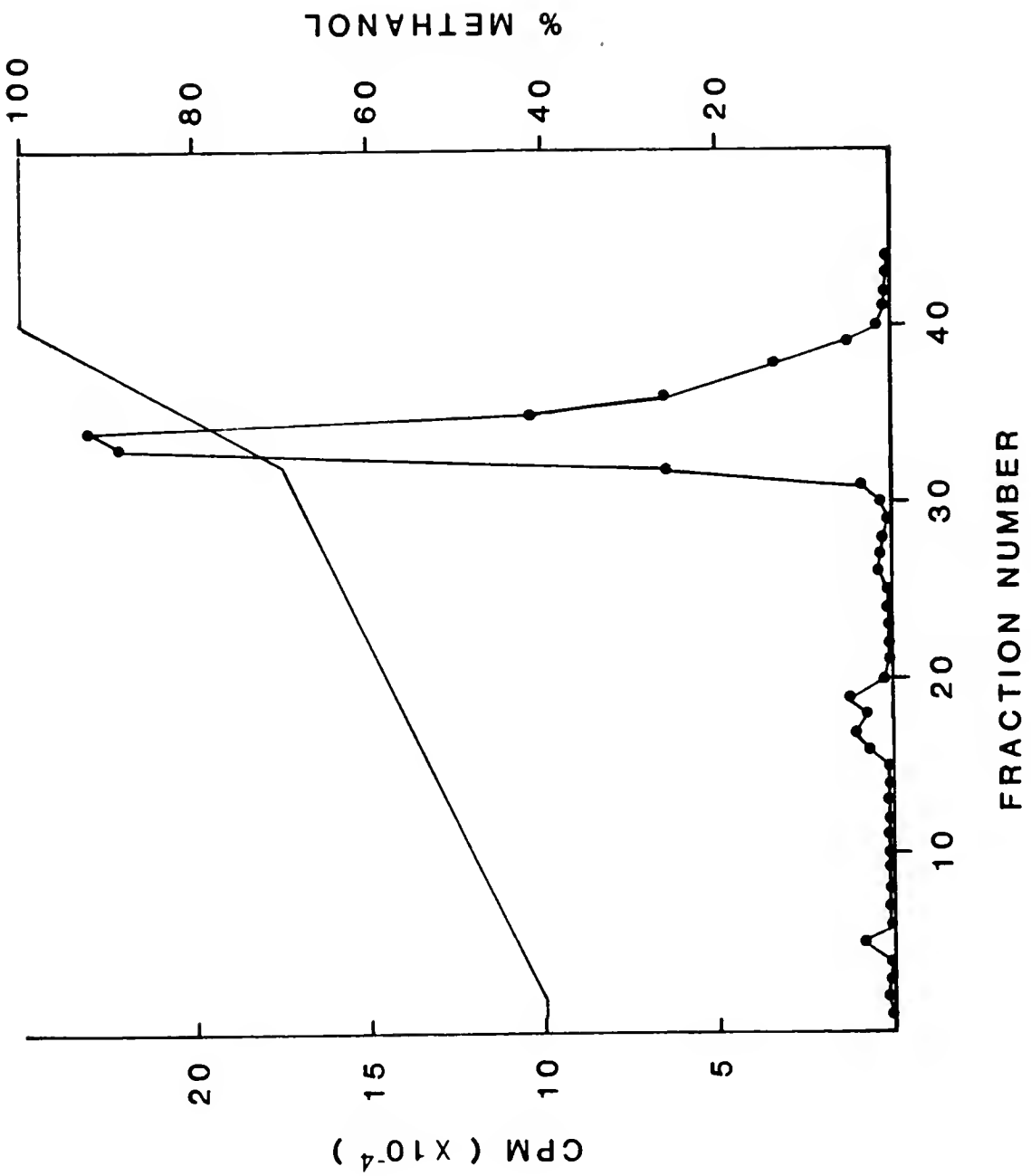
centrifugation at 30,000 x g for 20 minutes. The pellet was resuspended in phosphate buffer, and membranes were incubated at 10 mg/ml (wet weight) with inhibitors and ^{125}I-Tyr-bradykinin. The final reaction volume was 400 µl. After incubation of the reaction mixture was centrifuged and an aliquot of the supernatant was applied to a silica gel plate for chromatographic analysis. Chromatography was performed with a mixture of ethyl acetate:pyridine:water:acetic acid (65:18:9:4). In this system the intact peptide remained at the origin while major hydrolysis product migrated with an R_f value of 0.47. For degradation experiments in cultured brain cells, ^{125}I-Tyr-bradykinin was incubated with each culture in 5 mM potassium phosphate (pH 7.2) containing 320 mM sucrose. Integrity of the incubated radioactive ligand was determined by chromatography on cellulose plates and a solvent system of butanol:acetic acid:water (25:4:10, R_f = 0.5). The second system (butanol:acetic acid:water) was used to identify any hydrolysis products which might remain at the origin in the first system.

Results

Iodination

The dilute chloramine T method consistently yielded ^{125}I-Tyr-bradykinin of high specific activity (500-800 Ci/mmole) and purity (greater than 90% as determined by HPLC, Figure 15). The degree of iodination appeared to be most dependent on the "age" of the Na^{125}I. Fresh deliveries of Na^{125}I from the manufacturer gave higher specific activities than did older lots which had been used previously.

FIGURE 15: Elution profile of ^{125}I-Tyr-bradykinin. The radioactive peptide was eluted on a methanol:10 mM ammonium acetate gradient as shown in the figure. The radioactive peak corresponds to the peak elution of "cold" Tyr-bradykinin as indicated by an absorbance peak at 230 nM. Purity from this preparation was 93%.


% METHANOL
100
80
60
40
20
CPM (X10⁻⁴)
20
15
10
5
10
20
30
40
FRACTION NUMBER

Degradation studies in brain homogenate

A search for adequate inhibitors of brain kininases was designed along two strategies. First, conditions were used which might inhibit kininase activity, but which would probably not inhibit a receptor binding (Figure 16). Second, another set of conditions was designed which would certainly destroy enzymatic activity but would probably also inhibit receptor binding (Figure 17). This second set of conditions would assure that enzymatic degradation was present, and that it could be inhibited. Figure 16 demonstrates that Captopril, EDTA, dithiotheitol, bacitracin, trasylol, ovomucoid trypsin inhibitor and pH 8.9 were ineffective in inhibiting degradation, while of 1 mM 1,10-o-phenanthroline inhibited 40% of degradation. Higher concentrations of phenathroline were compared to severe conditions such as 0.1 M HCl and pretreatment of the tissue by boiling (Figure 17). Fifty and 100 mM 1,10-o-phenanthroline completely inhibited degradation, as did boiling and acid, but Triton X-100, DTNB and trypsin did not. A dose-response experiment (Figure 18) shows that a concentration as low as 5 mM phenanthroline was effective in blocking kinin degradation at 4° C for five minutes. Since the radioactive ligand would be required to be in the presence of the tissue for more than five minutes in a receptor assay the ability of 1,10-o-phenanthroline to inhibit degradation for one to two hours was assessed. Figure 19 shows that 5 mM phenanthroline completely inhibited degradation of ^{125}I-Tyr-bradykinin for one- and two-hour incubations.

Degradation studies in brain cell culture

Characterization of kinin receptors required the use of cultured brain cells to decrease non-specific absorption (see Chapter V). Thus,

FIGURE 16: Percent ^{125}I-Tyr-bradykinin (BK) remaining intact after incubation with rat brain homogenate. ^{125}I-Tyr bradykinin was incubated with untreated membranes or with membranes treated with various inhibitors or at different pH. Reactions were carried out for five minutes at 4° C. Membranes were centrifuged and an aliquot of the incubation buffer was spotted on a silica gel plate for chromatographic analysis. The plate was cut into one centimeter wide strips and counted in a gamma counter. Percent of the ligand remaining intact was determined by dividing the total cpm under the intact peak by the total cpm in the migration profile. MEMB, rat brain membranes; CAP, Captopril; EDTA, ethylenediamine tetracetic acid; PHEN, 1,10-o-phenanthroline; DTT, dithiothreitol; BAC, bacitracin; TRAS, trasylol; OMTI, ovomucoid trypsin inhibitor.

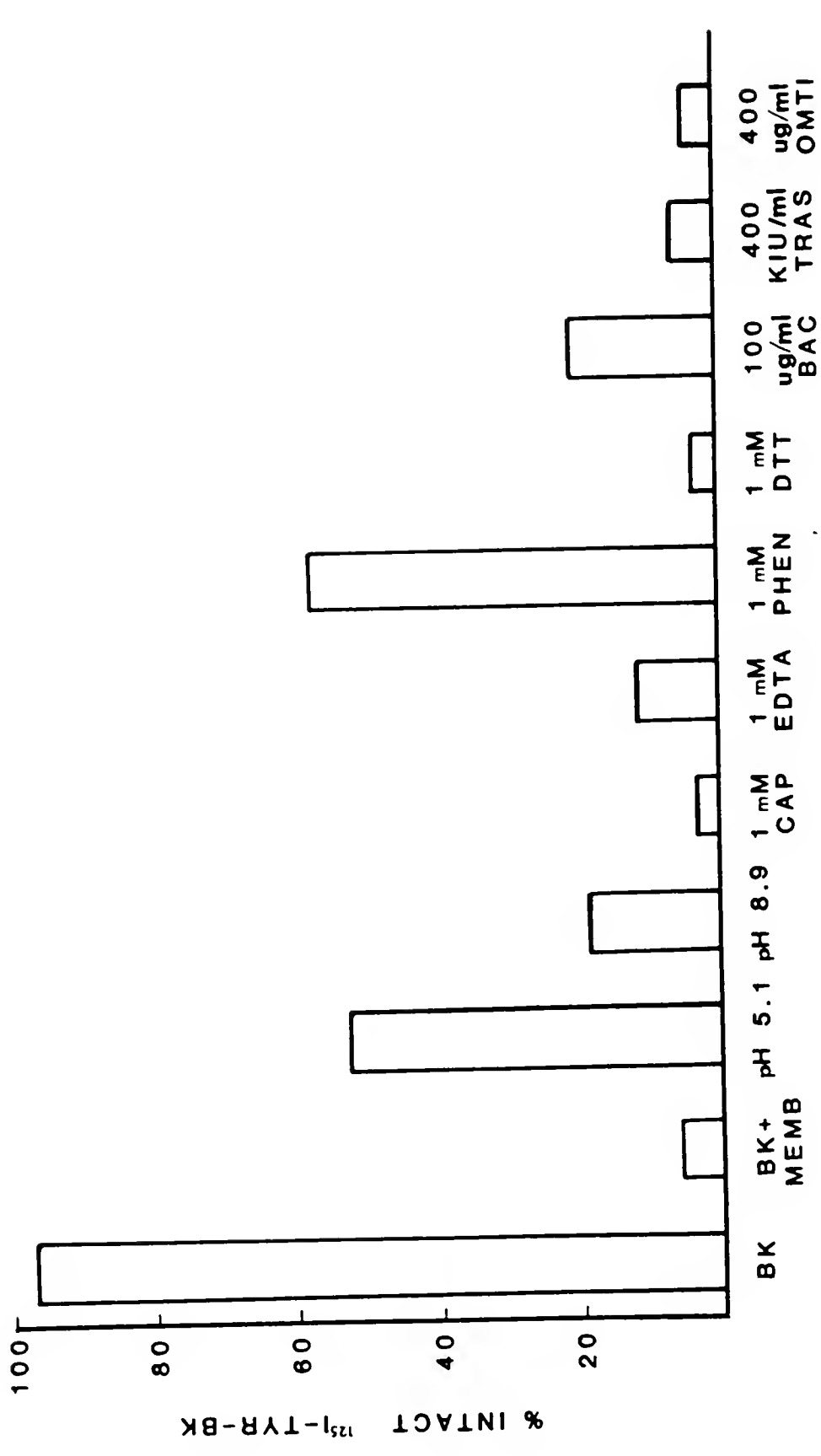

% INTACT ^{125}I-TYR-BK
100
80
60
40
20
BK
BK+ MEMB
pH 5.1
pH 8.9
1 mM CAP
1 mM EDTA
1 mM PHEN
1 mM DTT
100 ug/ml BAC
400 KIU/ml TRAS
400 ug/ml OMTI

FIGURE 17: Percent ^{125}I-Tyr-bradykinin (BK) intact following treatment with rat brain membranes (MEMB) or with membranes plus various inhibitors or low pH. For membranes which were pretreated by boiling, the tissue was placed in a boiling water bath for ten minutes prior to incubation with the radioactive ligand. All other conditions and methods of analysis were the same as in Figure 16. TRITON, Triton X-100; DTNB, dithiobis-2-nitrobenzoic acid; PHEN, 1,10-o-phenanthroline.

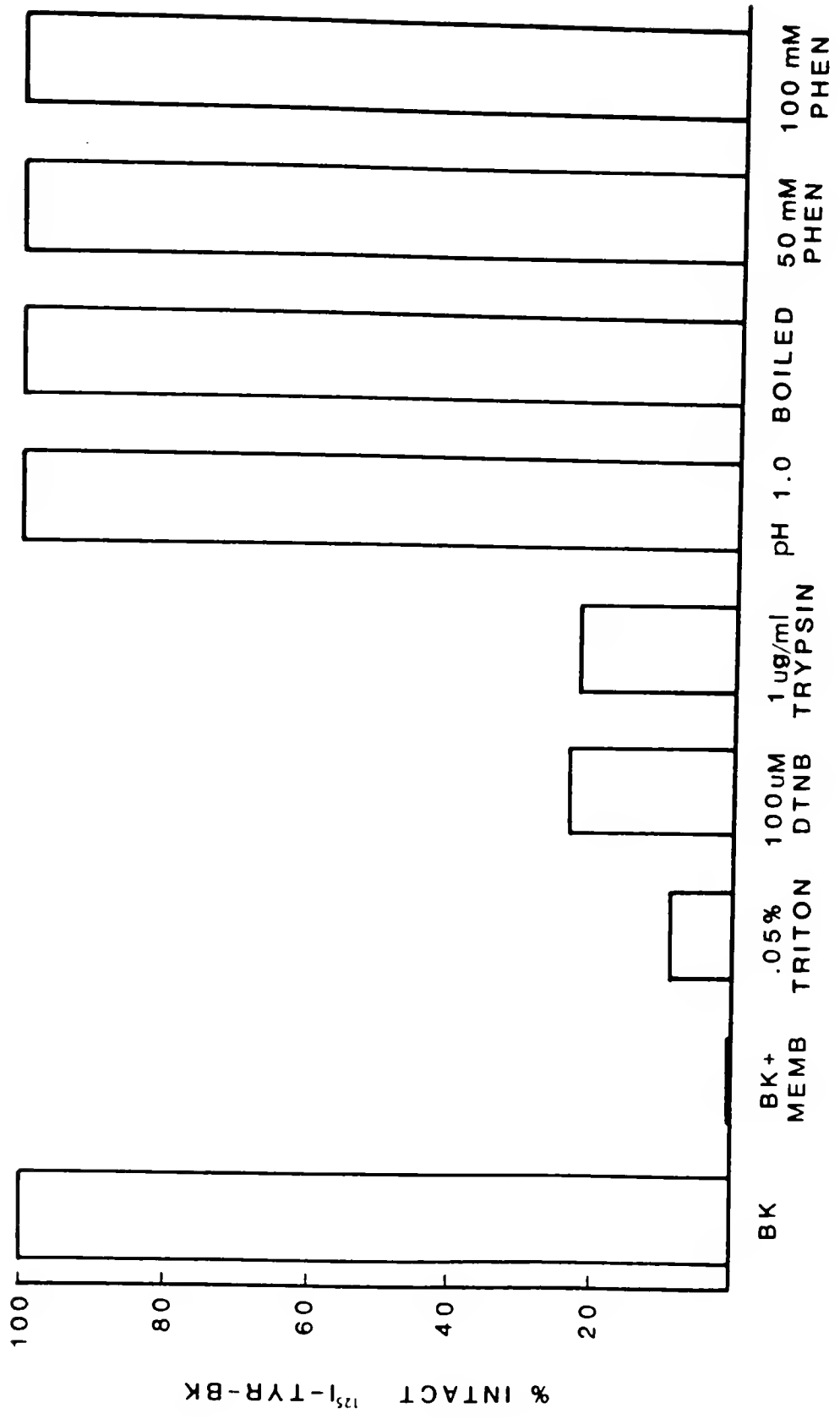

% INTACT ^{125}I-TYR-BK
100
80
60
40
20
BK
BK+ MEMB
.05% TRITON
100uM DTNB
1ug/ml TRYPSIN
pH 1.0
BOILED
50 mM PHEN
100 mM PHEN

FIGURE 18: Dose-response curve for phenanthroline inhibition of ^{125}I-Tyr-bradykinin. Incubations were carried out for five minutes at 4° C with different doses of 1,10-o-phenanthroline.

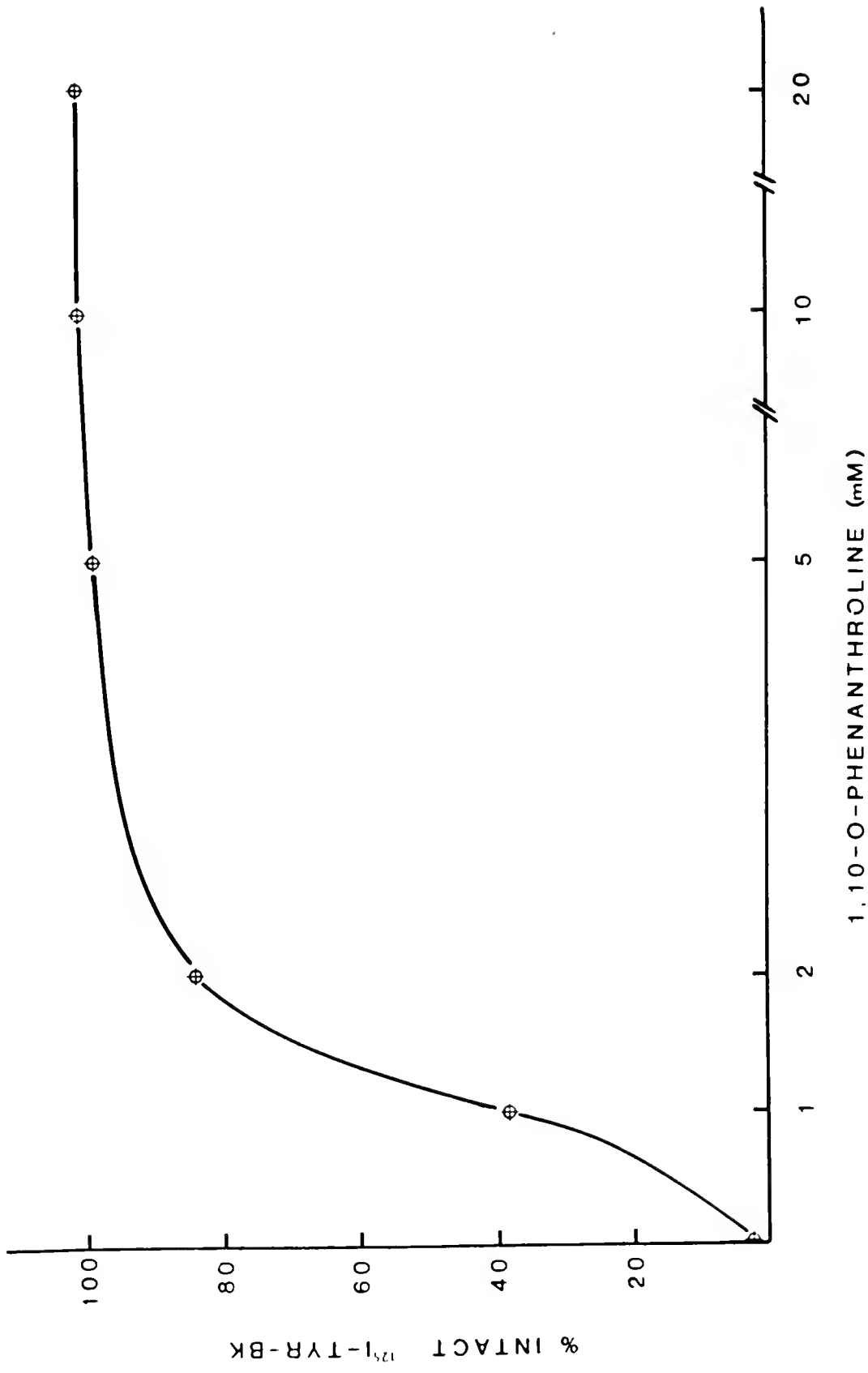
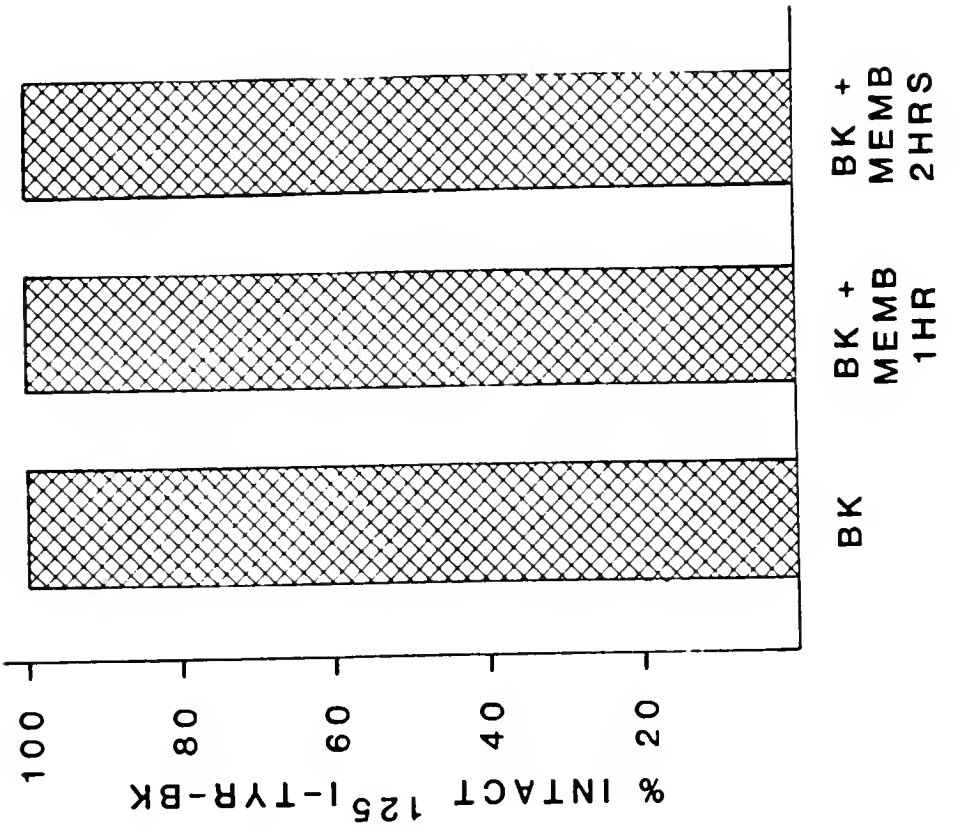

% INTACT ^{125}I-TYR-BK
100
80
60
40
20
1
2
5
10
20
1,10-O-PHENANTHROLINE (mM)

FIGURE 19: Inhibition of ^{125}I-Tyr-bradykinin (BK) degradation by 5 mM 1,10-o-phenanthroline. The radioactive ligand was incubated with rat brain membranes (MEMB) in the presence of 5 mM phenanthroline for 1-2 hours at 4° C. No degradation of the incubated ^{125}I-Tyr-bradykinin was observed.


% INTACT ^{125}I-TYR-BK
100
80
60
40
20
BK
BK + MEMB 1HR
BK + MEMB 2HRS

it was prudent to investigate the integrity of the iodinated ligand following incubation with these intact cells. Because whole cells in an isotonic medium will retain their intracellular enzymes and consequently decrease the potential for proteolytic degradation, the dose of 1,10-o-phenanthroline was decreased by half from the previous experiments to 2.5 mM. However, 2 μM SQ 20,881 was added to the incubation medium because subsequent experiments for characterization of kinin binding sites required assurance that the radioactive ligand was not bound to kininase II (Chapter V). Table 1 shows that two hours of incubation at 4° C resulted in only a 3% loss in intact ^{125}I-Tyr-bradykinin. Incubation of the ligand for two hours at room temperature caused slightly more degradation and a 24-hour incubation resulted in a 14% loss of ^{125}I-Tyr-bradykinin.

Discussion

The ultimate application of the radioactive kinin analogue is characterization of specific, high affinity kinin binding sites in CNS tissue. Proper choice of ligand is necessary to maximize receptor affinity. A variety of radioactive analogues of bradykinin have been prepared for use in binding studies and radioimmunoassay (25, 26, 162, 163). In these studies ^{125}I-Tyr-bradykinin (^{125}I-Tyr^{1}-kalladin) was selected as the radioactive probe for two reasons: biological activity and cost of preparation. Previous investigations (25) have shown Tyr-bradykinin and its iodinated form to retain almost all of their biological activity in smooth muscle preparations, whereas other iodinated kinin analogues with tyrosine substitutions at the 8 and 5

TABLE 1
Degradation of ^{125}I-Tyr-bradykinin in Cultured Brain Cells

Incubation conditions	Percent intact
Control	95.5 ± 0.7
2 hrs., 4° C	93.0 ± 0
2 hrs., 24° C	89.0 ± 4.0
24 hrs., 4° C	82.0 ± 1.0

positions have little or no biological activity in these same preparations (14, 25). Tyr-bradykinin is also active when injected into the brain (Figure 14) and thus is a suitable probe for investigating kinin receptors in the CNS.

Tritiated analogues of bradykinin have been used (26, 163) but these ligands must be purchased by commercial suppliers and are expensive. Iodinated kinins are preferrable because the molecule can be radioactivity tagged and purified in the laboratory by simple procedures for relatively little cost. Additionally, the specific activity of the iodinated kinin is much greater than tritated kinins available from commercial sources (500-800 Ci/mmole vs 50 Ci/mmole, respectively).

The presence of kininases has been extensively reported in brain (93-95, 101, 107, 111-116) as well as other tissues which elicit biological activity in the presence of kinins (92, 164). Inhibition of degradation is a prerequisite to a successful receptor binding assay, without which receptor-ligand equilibrium cannot be achieved. A variety of enzyme inhibitors were unable to stop degradation of the radioactive ligand even at 4° C. The inability of Captopril to stop degradation suggests that the radioactive kinin is rapidly destroyed by enzymes other than kininase II (angiotensin-converting enzyme), an enzyme known to be present in various brain regions (93, 94). The metal chelator 1,10-o-phenanthroline is capable of binding metal ions often found as co-factors in enzymes. Since 5 mM phenanthroline was effective in blocking ^{125}I-Tyr-bradykinin degradation in brain homogenate for up to two hours at 4° C, the major proportion of kininase activity in cold brain tissue is through enzymes with metal co-factors. Phenanthroline

was equally effective in blocking degradation of kinins in cultured brain cells. The anatomical integrity of cultured cells keeps the majority of proteases sequestered within intracellular compartments and probably decreases the degradation potential of this preparation.

Selection of an appropriate ligand, and inhibition of degradation of that ligand in the proper tissue preparation should allow an accurate search for kinin binding sites in brain tissue.

CHAPTER V
SPECIFIC BINDING OF ^{125}I-TYR-BRADYKININ IN CULTURED RAT BRAIN CELLS

Rationale

Given the biological actions of exogenous kinins in vivo it is reasonable to postulate that the biological response occurs by kinins binding to these receptors. Binding sites have been identified in brain tissue for a variety of peptides (159, 160, 165, 166), but kinin binding sites have not previously been identified in CNS tissue and have only recently been described in peripheral tissue (25, 26, 163, 164). Binding studies in CNS tissue tend to amplify the technical problems normally found when other tissues are utilized. The high lipid content of brain may contribute to the high levels of nonspecific binding, and the high levels of protease activity can cause rapid degradation of the radioactive ligand. The methods described in Chapter IV will be combined with brain cell culture to overcome these technical problems.

Methods

Brain cell culture

Neonatal rats were obtained from Sprague-Dawley rats mated within the laboratory. Pregnant Wistar-Kyoto and spontaneously hypertensive rats were obtained from University of Florida animal resources. Cells from neonatal rat brain were cultured according to the methods of Razaida et al. (165) with minor modifications. All procedures were

performed aseptically. Neonates of 0-1 day in age were placed in an isotonic salt solution (136 mM NaCl, 5.4 mM KCl, 0.16 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 5.5 mM glucose and 59.0 mM sucrose) containing 100 units penicillin G, 100 µg streptomycin and 0.25 µg Fungizone (Gibco) per ml, pH 7.2. The cranium was opened, and the whole brain was exised at the level of the medulla and placed in the isotonic salt solution. Under magnification each brain was cleaned free of pia mater and minced with iris scissors. The minced tissue was then incubated twice for 15 minutes at 37° C with a total volume of 0.25% trypsin (Worthington) in isotonic salt solution and 25 ml of trypsin added prior to each incubation. The mixture was constantly agitated. At the second incubation 160 µg DNAase I (Sigma) in isotonic salt solution was also added. Next, the dissociated cells were centrifuged at 800 x g for 5 minutes and the supernatant was aspirated. The pellet was triturated and suspended in 40 ml Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco) and filtered through sterile gauze into a sterile bottle. The cell suspension was diluted to the appropriate concentration and cells were pipetted onto sterile Falcon culture dishes. Mixed cultures containing both glia and neuronal cell types were plated on 35 mm dishes at a density of 2.8×10^6 cells/plate. Neuronally enriched cultures were plated at a density of 5×10^6 cells/plate on 60 mm culture dishes precoated with .001% poly-L-lysine. Cells were fed on day three after plating with DMEM containing 5% FBS and 10% horse serum (HS, Gibco). Successive feedings occurred at four-day intervals and consisted of DMEM containing 10% HS. Neuronally enriched cultures were treated with cytosine arabinoside (10 µM) on days 2 and 3 to minimize glial growth and proliferation in these cultures.

Measurement of ^{125}I-Tyr-bradykinin binding

The specific binding of ^{125}I-Tyr-bradykinin to membrane receptors was measured on intact brain cells attached to sterile plastic culture dishes. Prior to each assay cells were washed twice with small volumes of 5 mM potassium phosphate buffer (pH 7.2) containing 0.32 M sucrose. Cultures were typically incubated in triplicate for two hours at 4° C in 5 mM potassium phosphate/0.32 M sucrose containing 2.5 mM 1, 10-o-phenanthroline (Sigma), 2 μM SQ 20,881 (Penninsula, Bachem), and 0.3 nM ^{125}I-Tyr-bradykinin. For determination of nonspecific binding some plates also contained 0.1 - 0.2 μM unlabelled bradykinin or kinin analogues where indicated. Following incubation the plates were rapidly rinsed (<1 minute) three times with the ice cold potassium phosphate sucrose buffer. The monolayer in each dish was then dissolved with 0.5 ml of 2.0 M NaOH. The dissolved tissue was transferred to 12 x 75 cm test tubes, the plates were rinsed once with 0.5 ml water and this was pooled with the original sample. Samples were counted in a Beckman gamma 5500 counter (74% efficiency). Specific binding was determined by subtracting cpm bound in the presence of excess unlabelled kinin from cpm bound in cultures without unlabelled peptide added. Protein was determined by the method of Lowry et al. (167) from samples used in each experiment or from identically grown cells. Biphasic competition curves were fitted according to IC_{50} values and the percentage of the high affinity sites calculated by an iterative program based on a one- or two-site receptor model(168).

Results

Morphology

Cultures contained cells having morphology consistent with that of glia and neurons. In developed cultures the larger glial cells formed a flat monolayer on the bottom of each plate upon which neurons attached and developed. Neurons were distinguished by their extensive neurite development. A photograph of a typical culture is shown in Figure 20.

Characteristics and specificity of binding

Figure 21 shows that binding to these cultures is linear in proportion to cell number within the range examined. Typical experiments were carried out at cell densities of 2×10^4 cells/cm^2 in 35 mm dishes, and corresponded to approximately 300 µg of protein per dish. Optimum pH was within the neutral range (pH 7.2-7.5, Figure 22), so subsequent experiments were carried out at pH 7.2.

Incubation of ^{125}I-Tyr-bradykinin with whole brain cells resulted in a time dependent increase in specific binding at 4° C (Figure 23), reaching a maximum in two hours. In subsequent experiments no increase in specific binding was observed at ten hours association as compared to two hours. Nonspecific binding was stable and did not change significantly beyond five minutes. For all experiments specific binding (i.e., total amount bound minus the amount bound in the presence of excess unlabelled ligand) ranged between 35 and 40 percent of total binding. Five percent of the total radioactivity added (typically 200,000 cpm) was bound to cultures in the absence of unlabelled ligand.

Saturation experiments plotted by Scatchard analysis suggested two distinct high affinity components to ^{125}I-Tyr-bradykinin binding

FIGURE 20: Fetal rat brain cell culture. Note two distinct cell morphologies. Larger, flat cells are identified as glia cells. Cells with extensive neuritic processes on top of the glial monolayer are identified as neurons.

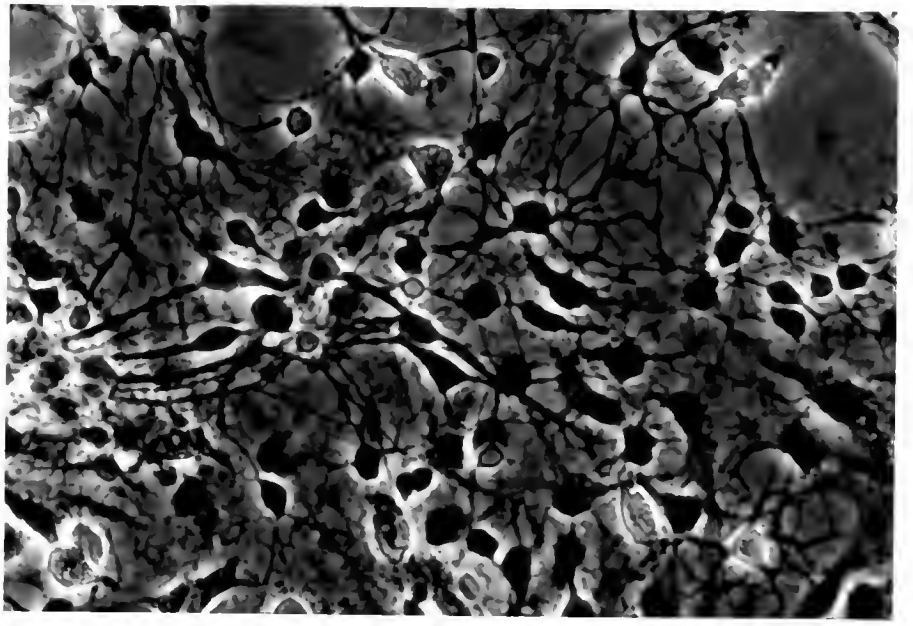


FIGURE 21: Specific ^{125}I-Tyr-bradykinin (^{125}I-Tyr-BK) binding is linear with increasing tissue concentration in culture. Varying densities of cells were grown in 60 mm plates. Cell density in subsequent assays was performed near 2 x 10^4 cells/cm^2, which corresponds to 300 µg of protein. Data are presented as mean ± S.E. of triplicate determination.

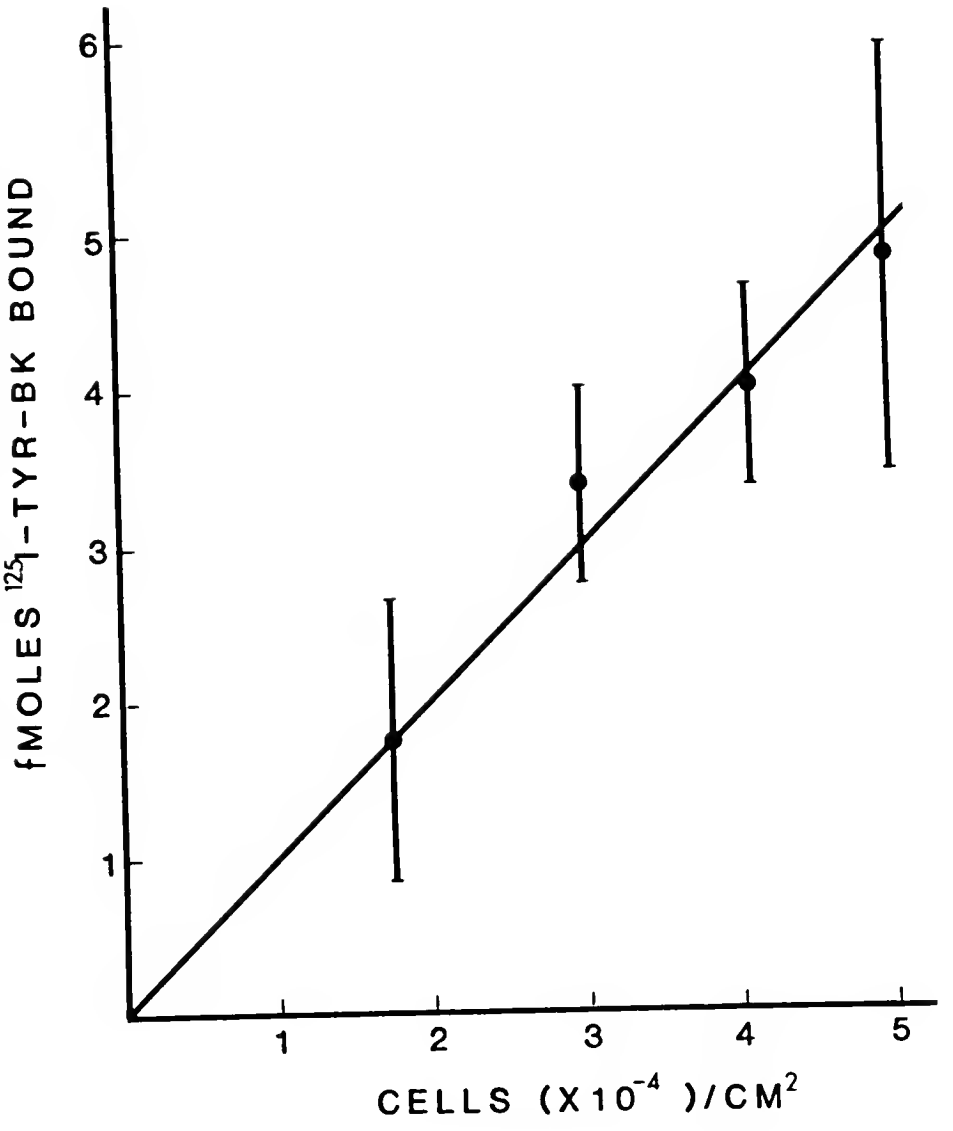

fMOLES ^{125}I-TYR-BK BOUND
6
5
4
3
2
1
1
2
3
4
5
CELLS (X 10^{-4})/CM^2

FIGURE 22: Specific ^{125}I-Tyr-bradykinin(^{125}-Tyr-BK) binding in brain cell culture demonstrates a pH optimum in the neutral range (pH 7.2-7.5). Subsequent experiments were performed at pH 7.2. Data are presented as mean ± S.E. of triplicate determinations.

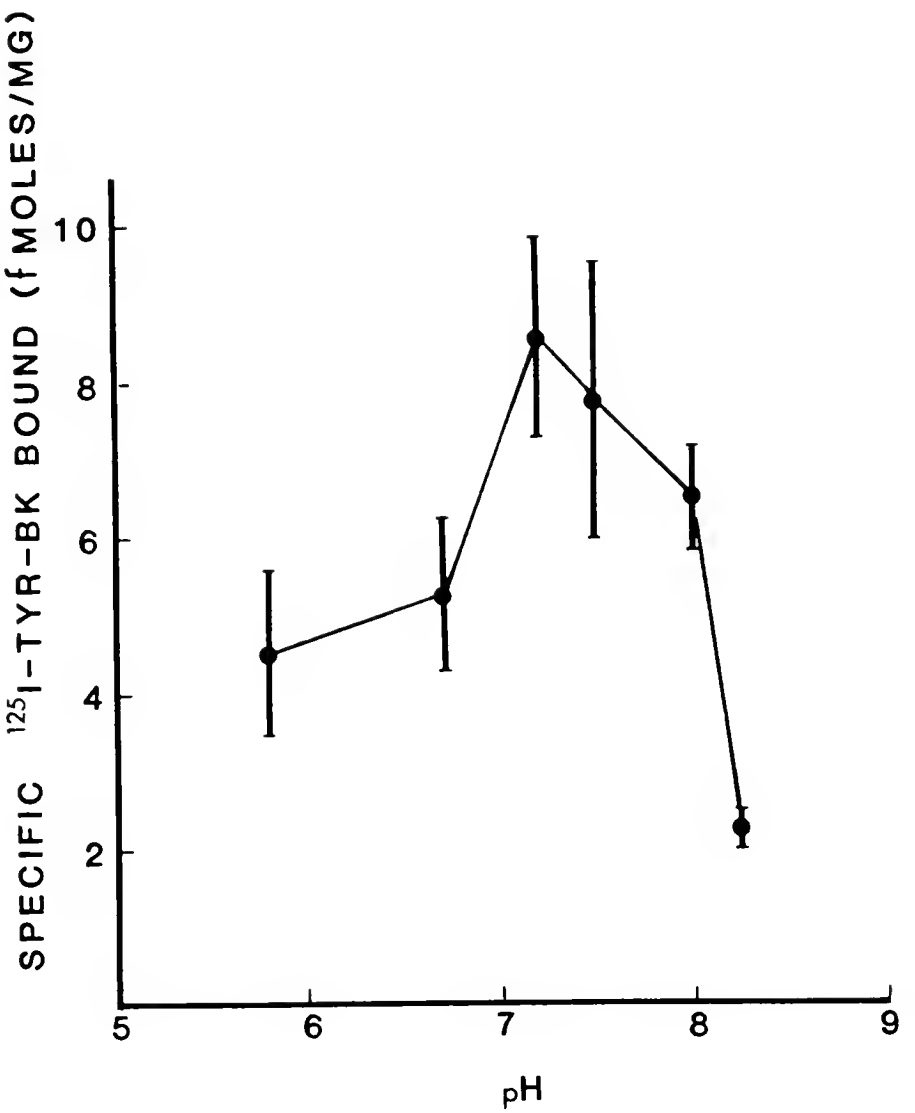
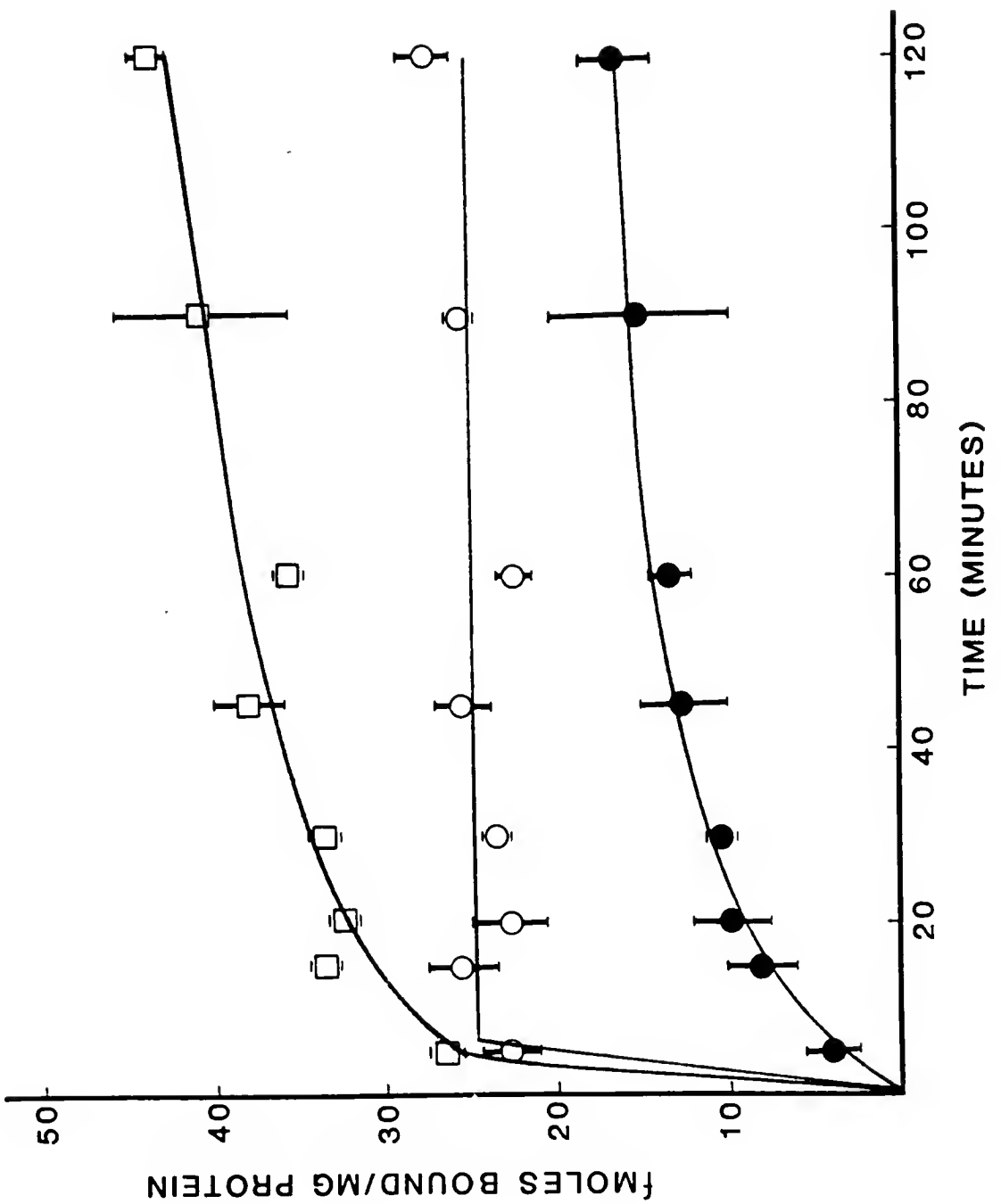

SPECIFIC ^{125}I-TYR-BK BOUND (f MOLES/MG)
10
8
6
4
2
5
6
7
8
9
pH

FIGURE 23: The association of ^{125}I-Tyr-bradykinin is time dependent. Identical cultures were incubated with 0.3 nM ^{125}I-Tyr-bradykinin alone (total binding, open squares) or in the presence of 0.2 µM unlabelled bradykinin (nonspecific binding, open circles). Incubations were terminated at the times illustrated by aspirating the incubate and washing rapidly (< 1 minute) three times with 5 mM potassium phosphate buffer containing 0.32 M sucrose (pH 7.2). Specific binding (closed circles) was determined by subtracting nonspecific binding from total binding.


fMOLES BOUND/MG PROTEIN
50
40
30
20
10
20
40
60
80
100
120
TIME (MINUTES)

in cultured cells. As shown in Figure 24 these components were resolved by linear regression into two lines with different slopes. For the higher affinity site the calculated K_D was 0.5 nM with a B_{max} of 72 fmoles/mg protein. The lower affinity site had K_D and B_{max} values of 21 nM and 1193 fmoles/mg protein, respectively. From this experiment the higher affinity sites represent only 6% of the total number of kinin binding sites.

When saturation experiments were done by competition for ^{125}I-Tyr-bradykinin binding with unlabelled kinin, displacement curves were biphasic when the competing kinin was added in concentrations greater than 0.2 μM. Figure 25 illustrates a biphasic competition curve with high affinity sites (0.9 nM K_D, 31% of total sites), and micromolar affinity sites (11 μM K_D, 69% of total sites) as determined by computer analysis. The micromolar affinity site appeared to be due to nonspecific, electrostatic attraction because binding in plates devoid of cells was displaceable when 10 μM, but not 0.2 μM, unlabelled bradykinin was added to the dishes.

Competition for radioligand binding was assessed with various kinin analogues, other unrelated peptides and prostaglandin E_1 (Figure 26 and Table 2). Most kinin analogues were potent in displacing binding with the order of potency Lys-bradykinin > bradykinin > Tyr-bradykinin > Tyr^8-bradykinin. Des-Arg^9-bradykinin did not compete for ^{125}I-Tyr-bradykinin at 1 μM or 10 μM. Other peptides (Table 2) displaced less than 10% of specific binding for high affinity binding at concentrations up to 1 μM.

FIGURE 24: Scatchard analysis of ^{125}I-Tyr-bradykinin binding. The experiment was performed by addition of increasing amounts of ^{125}I-Tyr-bradykinin alone or in the presence of 0.2 μM unlabelled Tyr-bradykinin in identical cultures. The plot was resolved into two components by linear regression. The higher affinity component had a K_D of 0.5 nM and B_{max} equal to 72 fmoles/mg protein. The lower affinity component revealed K_D and B_{max} values of 21 nM and 1193 fmoles/mg protein, respectively. Data are the means of triplicate determinations.

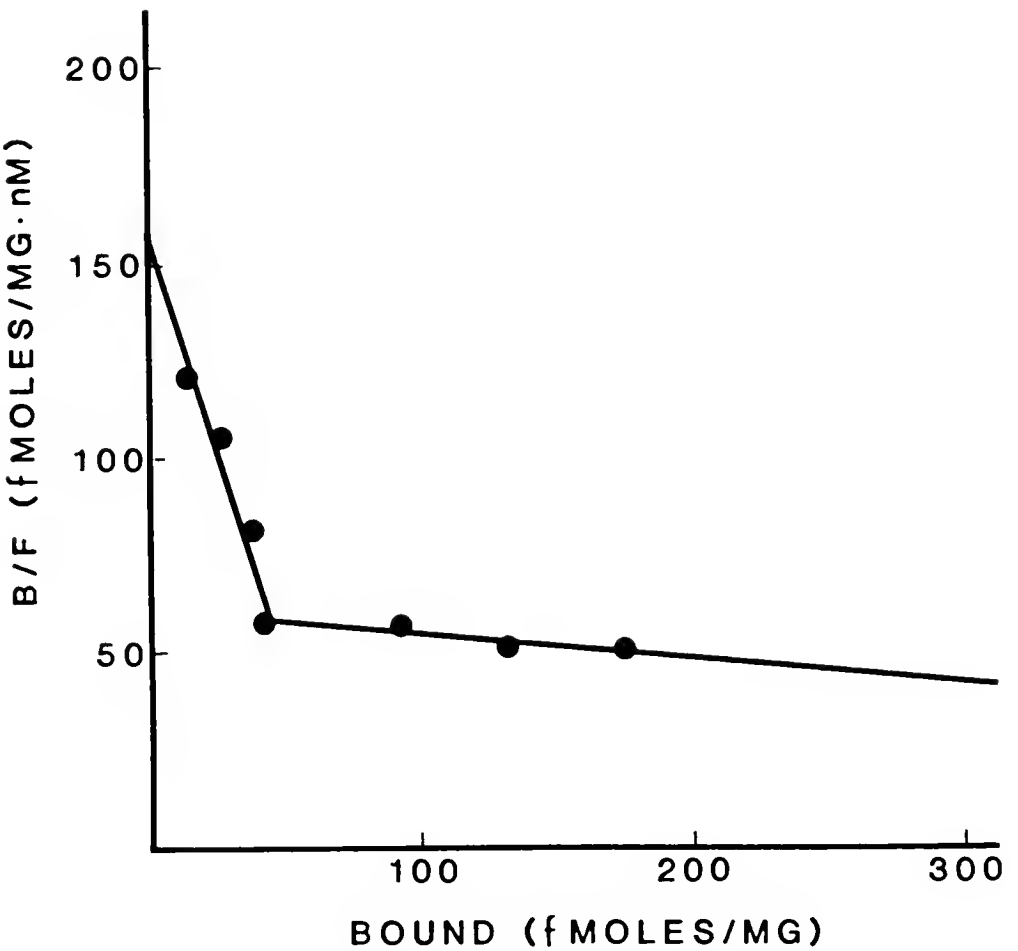

200
150
100
50
B/F (fMOLES/MG·nM)
100
200
300
BOUND (fMOLES/MG)

FIGURE 25: Concentrations of bradykinin greater than 0.2 μM displace ^{125}I-Tyr-bradykinin with low affinity and yield a biphasic competition curve. The experiment was performed by incubating 0.2 nM ^{125}I-Tyr-bradykinin with varying concentrations of unlabelled bradykinin. The right ordinate shows total counts bound. The left ordinate shows percent of total bound with zero percent bound taken as the amount of ^{125}I-Tyr-bradykinin displaced by 10^{-4} M of the competing ligand. Curves were drawn following analysis by an iterative computer program based on a two-site receptor model (168). Data are the mean ± S.E. of triplicate determinations.

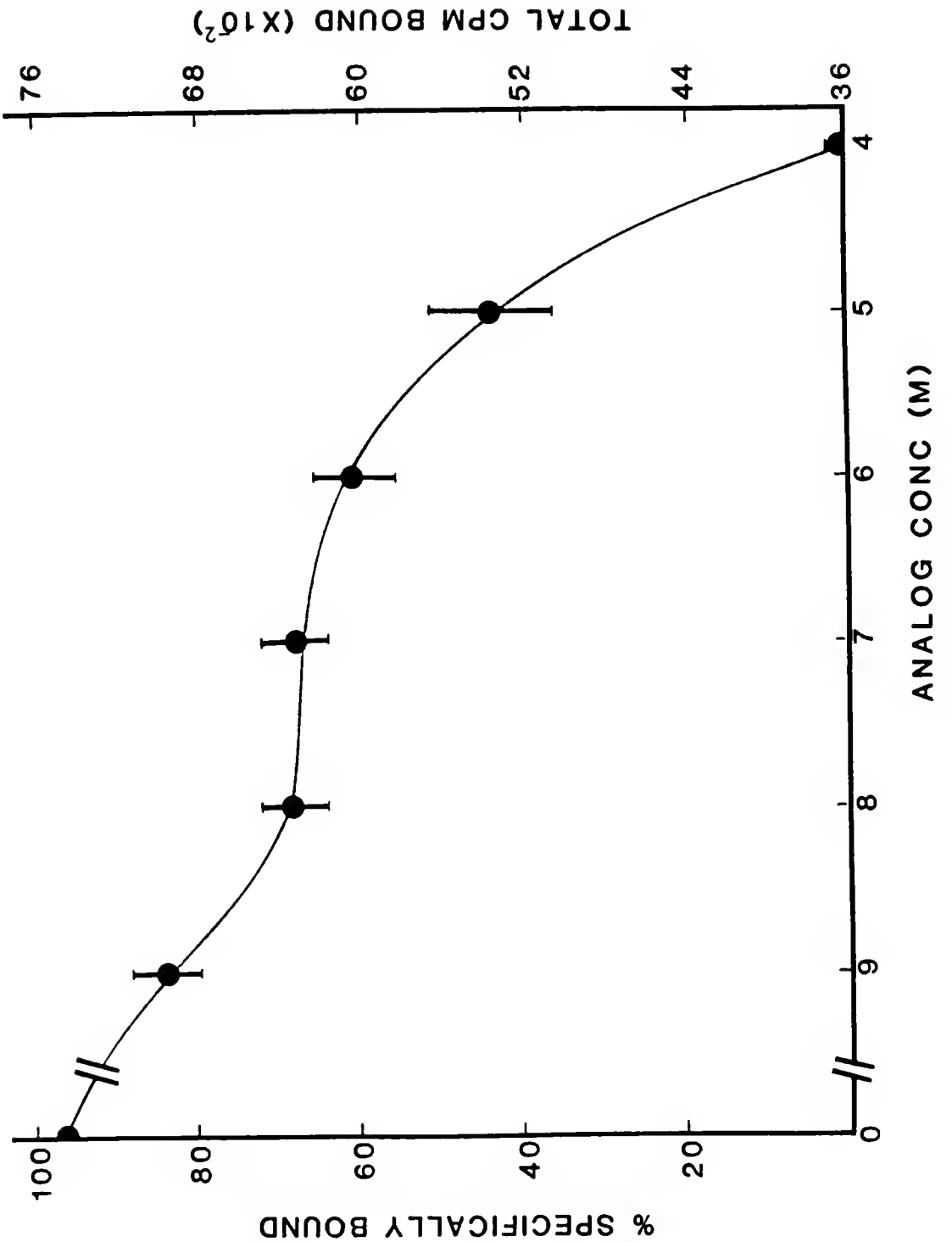

% SPECIFICALLY BOUND
100
80
60
40
20
0
TOTAL CPM BOUND (X10^2)
76
68
60
52
44
36
4
5
6
7
8
9
ANALOG CONC (M)

FIGURE 26: Kinin analogues compete with high affinity for ^{125}I-Tyr-bradykinin. Experiments were performed by incubating 0.3 nM ^{125}I-Tyr-bradykinin with varying concentrations of kinin analogues in identical cultures. Curves were drawn to correspond to the IC_{50} for each competing ligand. The doses of competing ligand were chosen to displace high affinity binding only. The order of potency is Lys-bradykinin (closed circles) > bradykinin (open circles) > Tyr-bradykinin (closed squares) > Tyr^{8}-bradykinin (open squares). Des-Arg^{9}-bradykinin (stars) did not compete for ^{125}I-Tyr-bradykinin binding at concentrations up to 10 μM. Data represent the means of triplicate determinations.

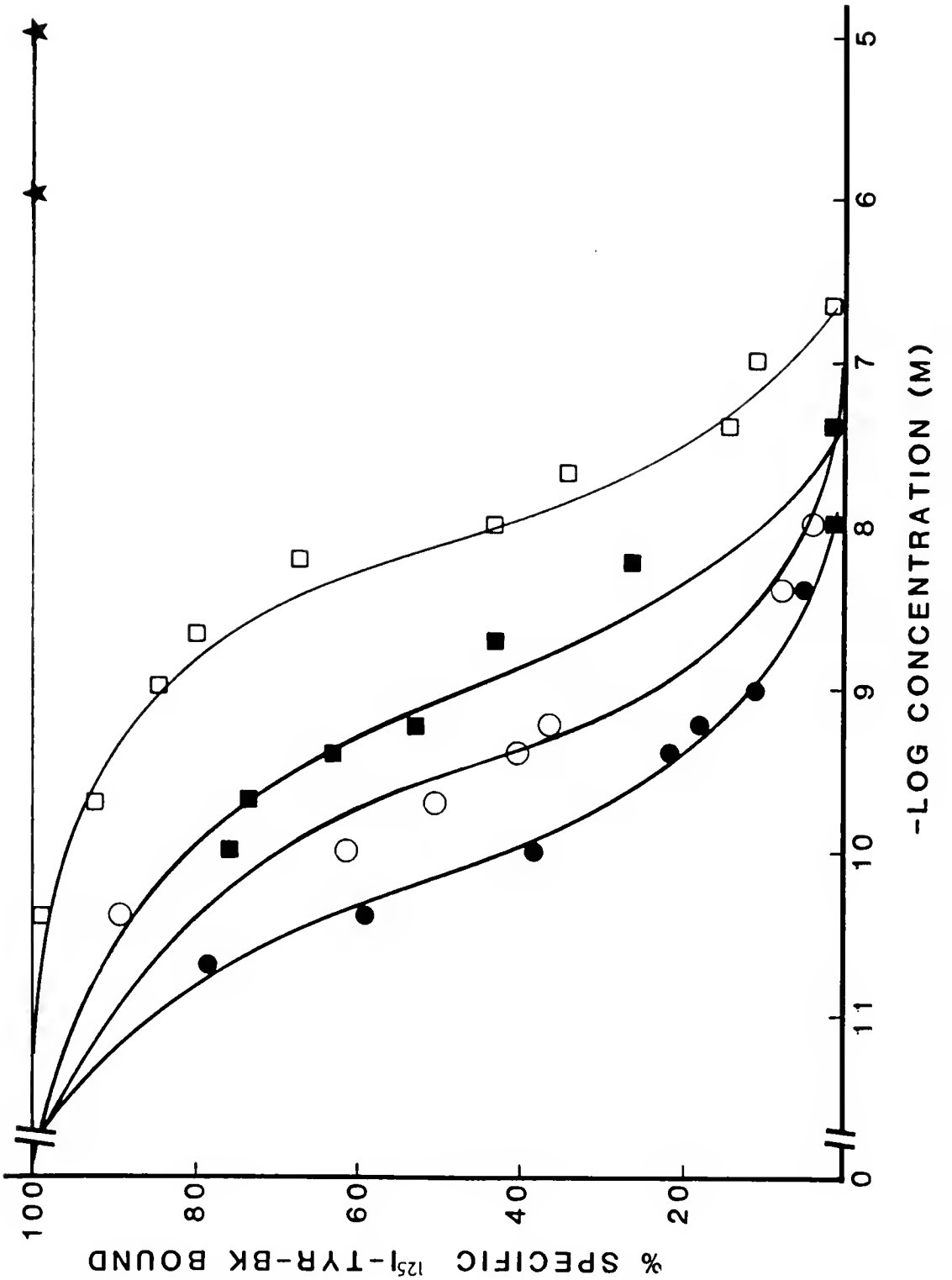

% SPECIFIC ^{125}I-TYR-BK BOUND
100
80
60
40
20
0
5
6
7
8
9
10
11
-LOG CONCENTRATION (M)

TABLE 2
Competition of Kinins and Unrelated Peptides
for ^{125}I-Tyr-Bradykinin Binding

Peptide	IC_{50} (nM)
Lys-bradykinin	0.07
Bradykinin	0.26
Tyr-bradykinin	0.86
Tyr^8-bradykinin	8.1
Des-Arg^9-bradykinin	$>10^{-5}$
Angiotensin II	$>10^{-6}$
Angiotensin I	$>10^{-6}$
Saralasin	$>10^{-6}$
Oxytocin	$>10^{-6}$
Met-enkephalin	$>10^{-6}$
Arginine vasopressin	$>10^{-6}$
Neurotensin	$>10^{-6}$
SQ 20,881	$>10^{-6}$
PGE_1	$>10^{-6}$

Reversibility experiments were performed by allowing ^{125}I-Tyr-bradykinin to bind to cells alone or in the presence of 0.2 μM unlabelled bradykinin for two hours, washing three times with incubation buffer, and then adding 2 ml fresh incubation buffer (containing phenanthroline and SQ 20,881). Dissociation was terminated at various times by removal of the incubate. This infinite dilution method yielded a biphasic semi-log plot (Figure 27). When the curvilinear plot was resolved into two components by linear regression dissociation rate constants ($k_{-1}=0.693/t_{1/2}$) of 1.3×10^{-4} sec^{-1} and 1×10^{-5} sec^{-1} were calculated. When k_{-1} is divided by the association rate constant k_1 (9.5×10^7 min^{-1} M^{-1}) dissociation constants of 0.76 pM for the rapidly dissociating component and 0.08 pM for the more slowly dissociating component are calculated. These kinetic values were several hundred-fold higher in affinity than those estimated by Scatchard analysis, so the possibility that the radioactive ligand was internalized or bound to "desensitized" receptors was considered.

To explore this possibility, ^{125}I-Tyr-bradykinin was incubated with cells for either two or ten hours and then dissociated by infinite dilution. If internalization of irreversible binding of the ligand was occuring, an increase in the length of association might alter the dissociation characteristics. However, no difference in the rate of dissociation was found (Table 3) with this manipulation. Consequently, the discrepancy between Scatchard and kinetic analysis was due to the extremely slow dissociation rates observed in Figure 27, since association rates were close to those expected for ligands with nanomolar affinity.

FIGURE 27: Dissociation of specifically bound ^{125}I-Tyr-bradykinin was carried out by allowing the radiolabelled ligand to associate with cultures for two hours at 4° C, washing the culture rapidly with incubation buffer and adding 2 ml incubation buffer to dissociate bound ^{125}I-Tyr-bradykinin by infinite dilution. A semi-log plot the data could be resolved into two components by linear regression. The faster dissociating component had a half-life of 88 minutes. The half-life of the slower dissociating component was 17 hours. Data are the mean ± S.E. of triplicate determinations.

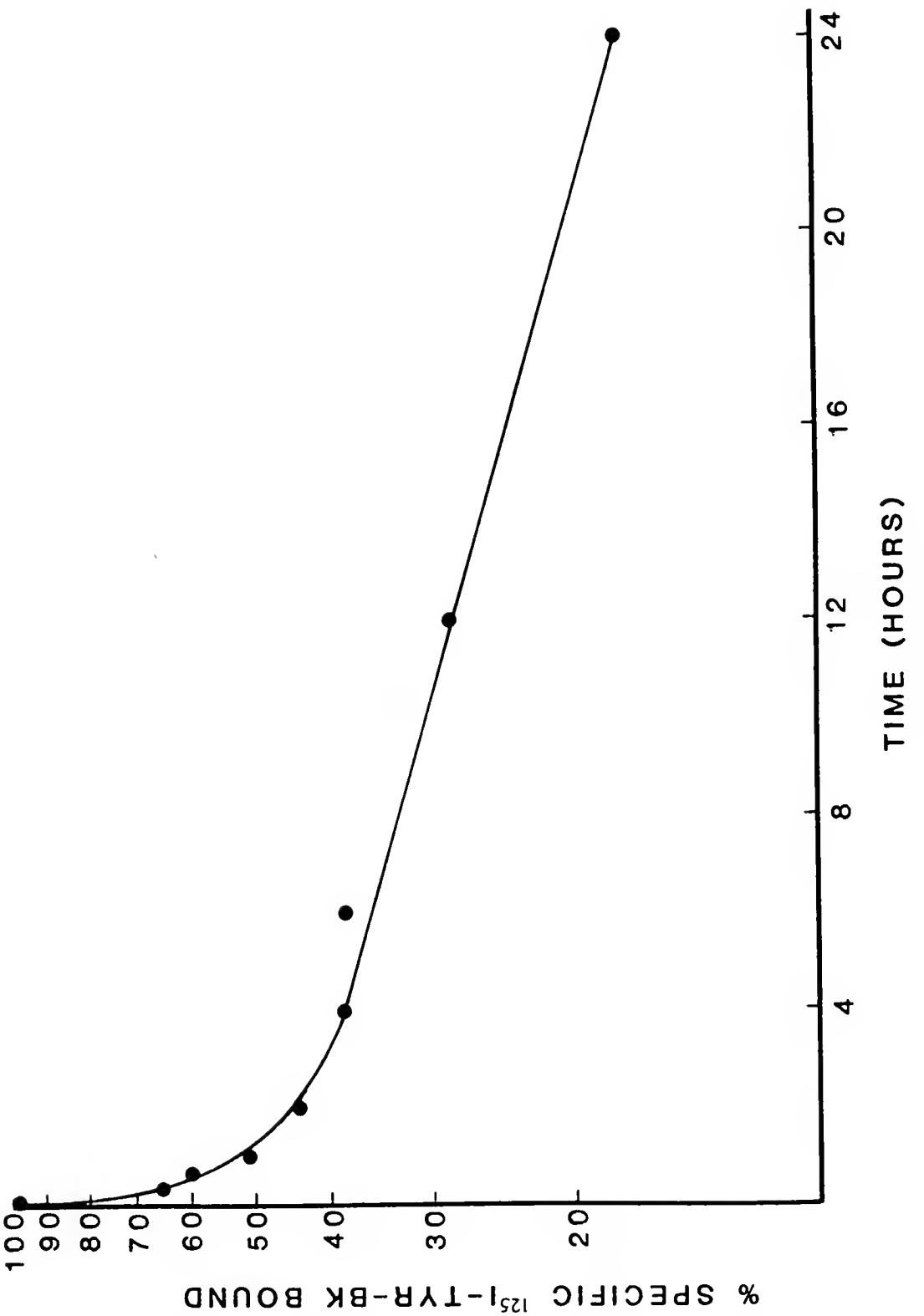

% SPECIFIC ^{125}I-TYR-BK BOUND
100
90
80
70
60
50
40
30
20
4
8
12
16
20
24
TIME (HOURS)

TABLE 3
Dissociation of ^{125}I-Tyr-bradykinin
following 2-Hour or 10-Hour Association

	% Bound	
	2-Hour Association	10-Hour Association
12-hour dissociation	46 ± 3%	44 ± 4%
16-hour dissociation	43 ± 5%	48 ± 9%

Monovalent and divalent cations (chloride salts) have potent effects on ^{125}I-Tyr-bradykinin binding (Table 4). Sodium appears to be slightly more potent than potassium in decreasing ^{125}I-Tyr-bradykinin binding, with IC_{50} values between 10 mM and 20 mM. Divalent cations also inhibited binding. Manganese was most potent, causing greater than 90% inhibition at 0.5 mM concentration. Both calcium and magnesium inhibit binding with IC_{50} values of approximately 1.0 mM.

Binding in different cell types

The relative proportion of glia and neurons can be manipulatd in culture. By performing experiments on cells with different ages in culture, in cultures treated with an antimitotic agent to kill glia and enrich the neuronal fraction, or in cultures which have been grown to confluency and can be passaged onto new plates to enrich the glial component, the relative contribution of different cell types can be assessed.

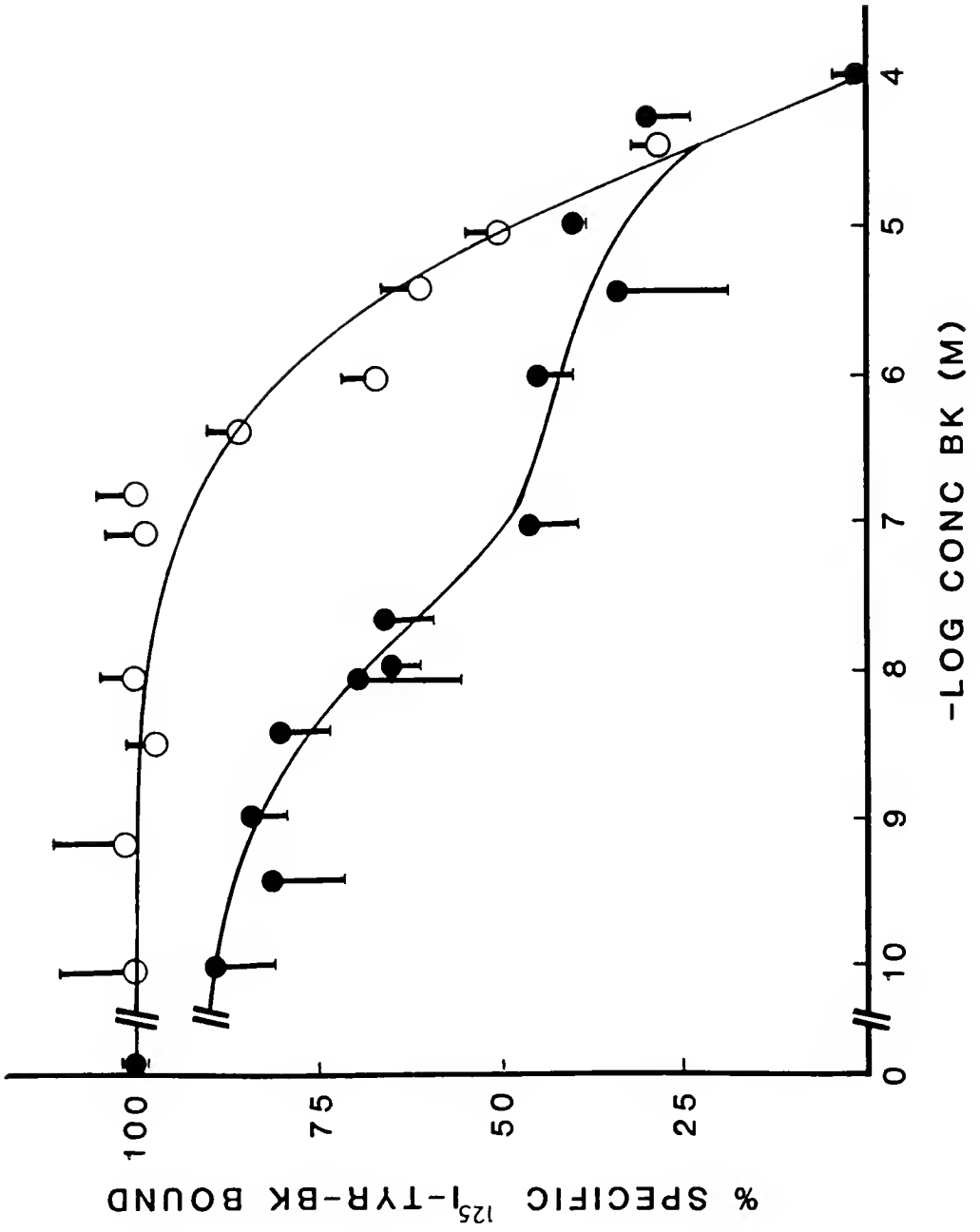
Differences in the binding properties of ^{125}I-Tyr-bradykinin were observed in young cultures containing cells with neuronal morphology. Figure 28 shows that six-day-old cultures retain the biphasic competition curve, the 54% of the sites being high affinity (4.2 nM K_D) and 46% of the sites being nonspecific (28 μM K_D) as calculated by computer analysis. However, 11-day-old cultures lack the high affinity site and demonstrate only nonspecific binding. From Figure 28 it is unclear whether the loss of high affinity sites in older cultures is due to an age-related process or to a low density of neurons in the older cultures. To discern between these possibilities, cultures

TABLE 4
Effects of Ions on ^{125}I-Tyr-Bradykinin Binding

% Specific Binding

Conc.	Monovalent			Divalent		
(mM)	Na^+	K^+	Choline	Ca^{++}	Mg^{++}	Mn^{++}
0	100±15	100±14	100±17	100±10	100±17	100±9
0.5					70±11	5±4
1				46±2	54±12	7±2
3				22±4	36±6	7±3
5				22±2	29±3	9±4
10	49±7	61±9		16±3	18±2	1±1
20	24±2	37±11				
40	25±7	31±12				
80	12±6	21±5				
120	10±3	14±9				
160	9±2	16±3	12±10			

FIGURE 28: Competition curves in six-day-old cultures (closed circles) and 11-day-old cultures (open circles) were compared. Curves in cultures of different age were drawn following analysis of the data by an iterative computer program. Zero percent bound was taken as the amount displaced by 10^{-4} M bradykinin (BK). Data are the mean ± S.E. of triplicate determinations.


% SPECIFIC ^{125}I-TYR-BK BOUND
100
75
50
25
0
10
9
8
7
6
5
4
-LOG CONC BK (M)

grown in cytosine arabinoside to kill mitotic glial cells and enrich the neuronal fraction were compared to glial-enriched cultures (Figures 29 and 30). Glial-enriched cultures in their second passage demonstrated only the nonspecific site (4.4 μM K_D), while neuron-enriched cultures demonstrated both high (0.6 nM K_D, 38% of total sites) and nonspecific (4.6 μM K_D, 62% of total sites) displacement.

Spontaneously hypertensive (SH) rats have an exaggerated pressor response to bradykinin (169). To explore the possibility that SH rats have altered binding characteristics saturation experiments were performed in cultures prepared from SH, Wistar-Kyoto (WKY) (genetic controls of SH rats) and Sprague-Dawley (SD) rat brains to examine the high affinity component (Figure 31). Scatchard analysis of the high affinity sites suggested a moderate increase in the affinity of kinin binding sites in SHR cultures compared to genetic control WKY cultures or to SD cultures (Figure 32). While SH cultures appeared to have fewer high affinity binding sites than WKY and SD, successive experiments make this relationship less clear (Table 5).

Summary

The major finding of this investigation was that ^{125}I-Tyr-bradykinin can label specific high affinity binding sites in cultured brain cells. In these cultured cells specific radiolabelled kinin binding averaged 40% of total binding when 0.1-0.2 μM of unlabelled kinin was used as the displacer. Higher concentrations of unlabelled kinin revealed a second low affinity site. To the extent that the competition with bradykinin was carried out, an IC_{50} for the low affinity site of greater than 10^{-6} M can be estimated. Since higher

FIGURE 29: UPPER: Neuron-enriched cultures prepared by growing cells in DMEM supplemented with 10% FBS and 10 μM cytosine arabinoside for 48 hours beginning three days after the initial plating. Note the high degree of neuritic development and absence of underlying glial elements.

LOWER: Glial-enriched cultures prepared by passaging brain cells to dilute out the non-mitotic neurons. The cells seen here are in their third passage.

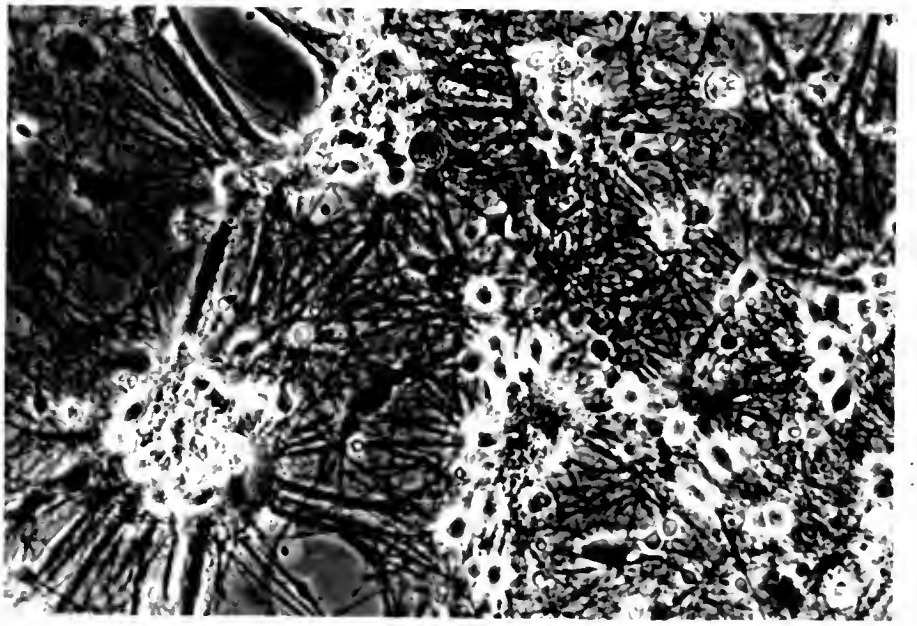


FIGURE 30: Comparison of competition curves with neuron-enriched (closed circles) or glial-enriched cultures (open circles). ^{125}I-Tyr-bradykinin (^{125}I-Tyr-BK) was competed for by varying concentrations of bradykinin. Curves were drawn following analysis of the data by an iterative computer program. Zero percent bound was chosen as the amount displaced by 10^{-4} bradykinin (BK). Data are the mean ± S.E. of triplicate determinations.

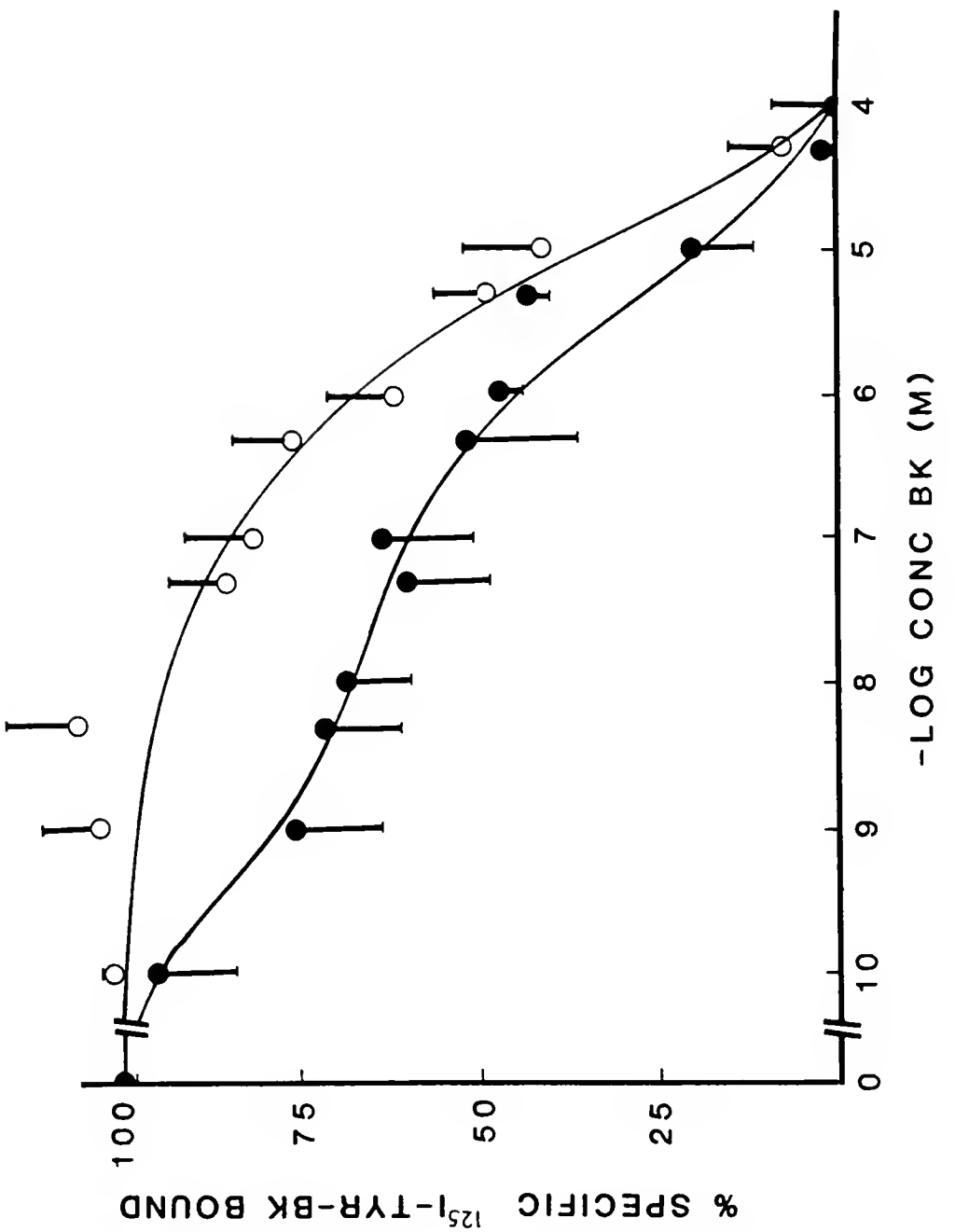

% SPECIFIC ^{125}I-TYR-BK BOUND
100
75
50
25
0
10
9
8
7
6
5
4
-LOG CONC BK (M)

FIGURE 31: Saturation curves for ^{125}I-Tyr-bradykinin (^{125}I-Tyr-BK) binding in cultures from the brains of SH rats (closed circles), WKY rats (open circles) or SD rats (closed squares). Experiments were performed by adding increasing concentrations of ^{125}I-Tyr-BK alone or in the presence of the unlabelled ligand. Data are the mean of triplicate determinations.

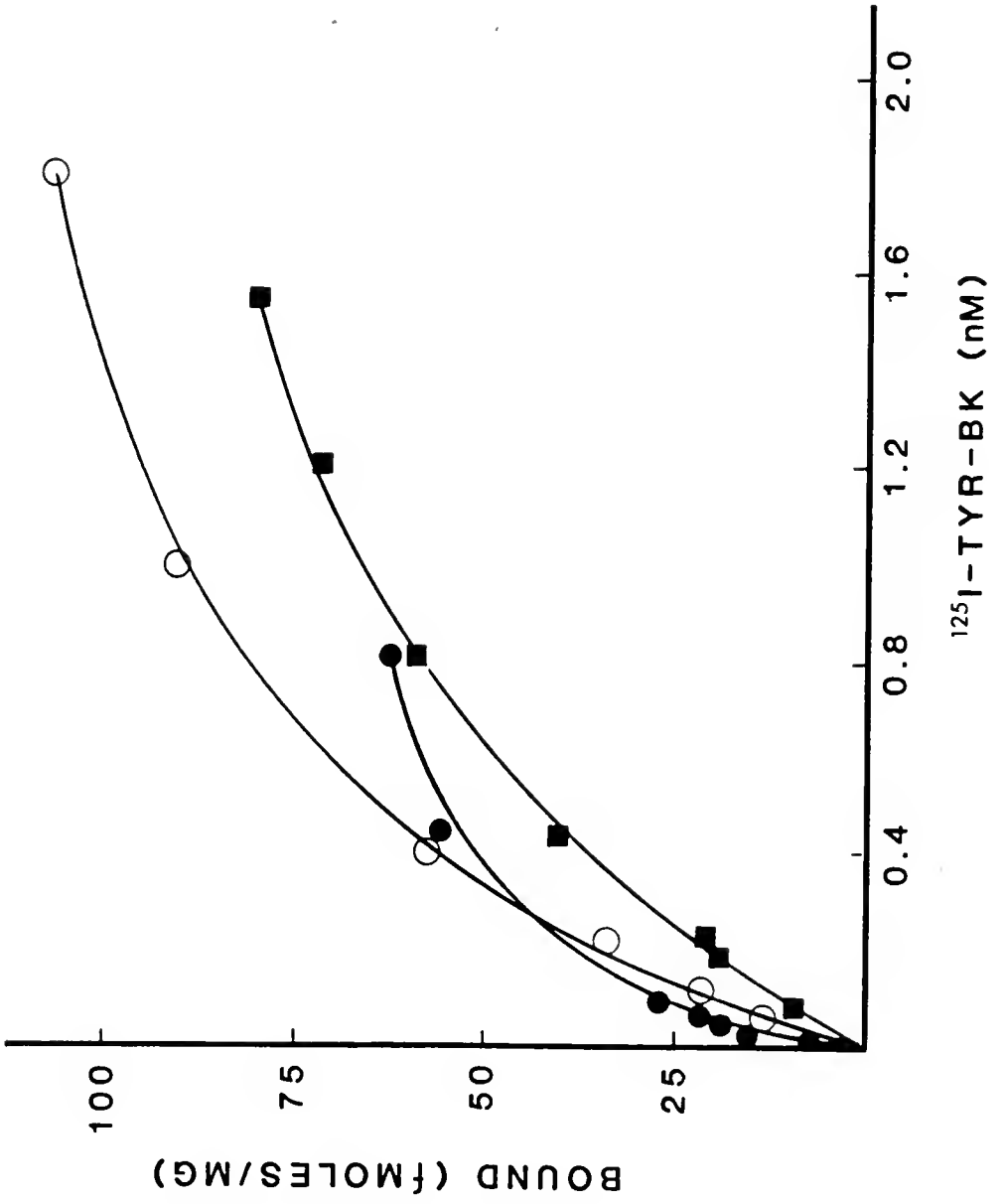

BOUND (fMOLES/MG)
100
75
50
25
0.4
0.8
1.2
1.6
2.0
^{125}I-TYR-BK (nM)

FIGURE 32: Scatchard analysis of saturation data plotted in Figure 31. Dissociation constant(K_D) and maximum number of binding sites are, respectively, 0.2 nM and 80 fmoles/mg protein for SH rats (closed circles); 0.7 nM and 153 fmoles/mg protein for WKY rats (open circles); and 1 nM and 134 fmoles/mg protein for SD rats (closed squares). Data are the mean of triplicate determinations.

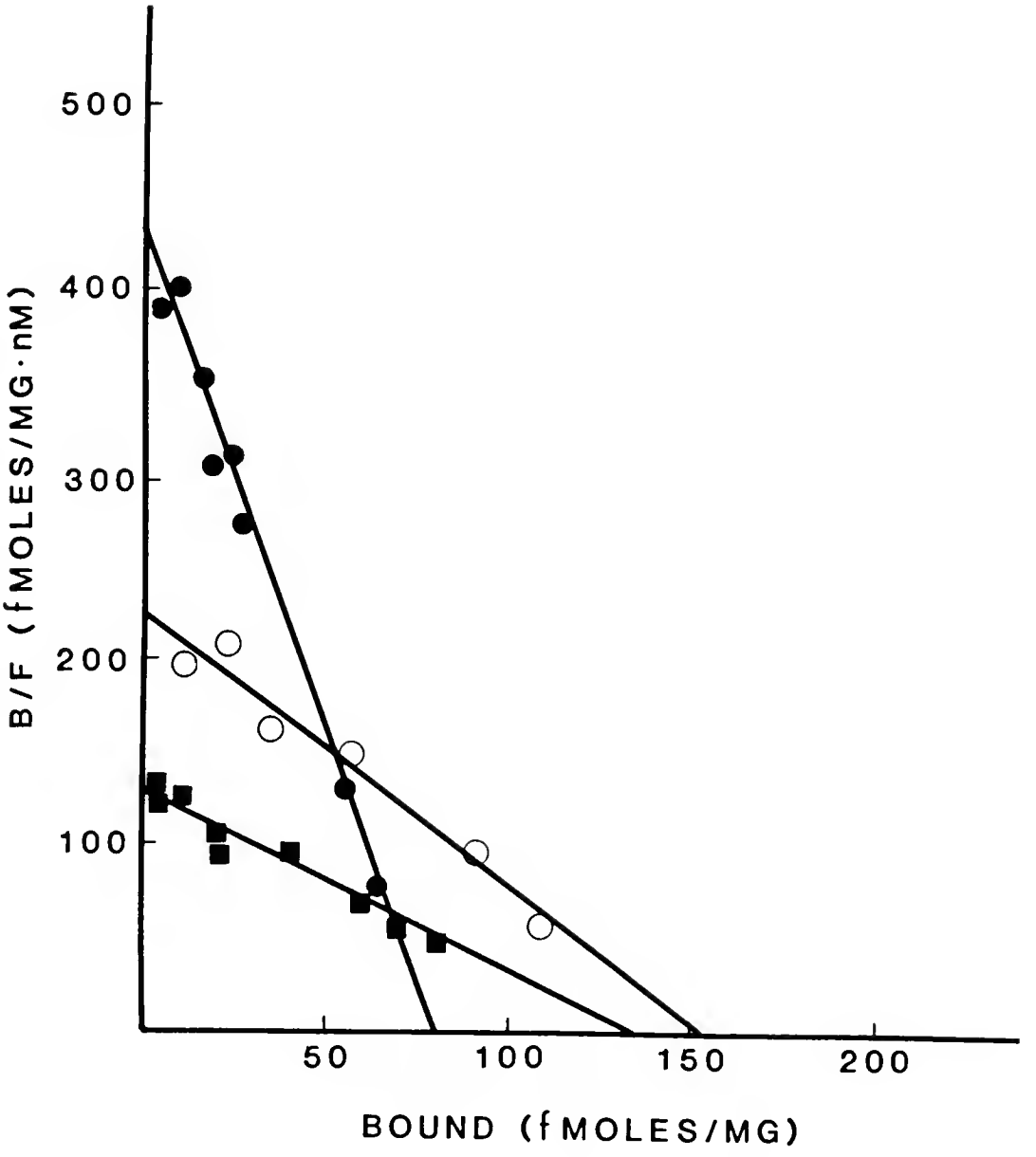

500
400
300
200
100
B/F (fMOLES/MG·nM)
50
100
150
200
BOUND (fMOLES/MG)

TABLE 5
Affinities and Maximum Binding Sites in Sprague-Dawley (SD), Spontaneously Hypertensive (SH) and Wistar-Kyoto (WKY) Rats

	High Affinity	
	K_D (nM)	B_{max} (fmoles/mg)
SD (N=3)	1.2 ± 0.6	103 ± 44
SH (N=3)	0.3 ± 0.1	78 ± 16
WKY(N=1)	0.7	153

concentrations of kinin ($>10^{-4}$) may lead to even more displacement of the radiolabelled ligand, the possibility that this low affinity site is no more than a nonspecific, electrostatic attraction must be considered. This appears plausible given the highly basic nature of bradykinin resulting from arginine residues at amino and carboxy terminals. A basic molecule in a relatively neutral buffer (pH 7.2) might be expected to have substantial attraction for negatively charged phospholipids on the cell's surface, or to the surface of the culture dish. Binding of the radiolabelled kinin to plates devoid of cells is displaced by 10 μM, but not 0.2 μM, cold bradykinin. This observation substantiates the idea that the low affinity site is not physiologically significant.

A pH optimum of 7.2 to 7.5 in cultured cells is in the range expected for a physiological receptor. The viability of the cultures at the different pH levels was not examined, but similar experiments in identical cultures failed to show a decrease in viability (165).

The order of potency for competing kinin analogues is consistent with what might be expected for a kinin formed in tissue. Kalladin (Lys-bradykinin), the most potent displacer, is the product of glandular kallikrein, the only known kininogen-specific enzyme in tissues. A tyrosine addition to the amino terminal decreases the ability of the peptide to bind, relative to bradykinin, approximately three-fold, and a tyrosine substitution for phenylalanine at position 8 lowers the competitive ability of the peptide approximately 30-fold compared to bradykinin. Removing the carboxy terminal arginine (Des-Arg^9-bradykinin) destroys all the ability of the peptide to compete for its

binding sites. Thus, the binding ability of bradykinin appears more sensitive to carboxy terminal changes than to alterations at the amino terminal. Furthermore, the order of potency for competing kinin analogues is consistent with classification of this binding site as a B_2 receptor, as classified by Regoli and Barabe (14). The B_2 receptor has high affinity for bradykinin but low affinity for Des-Arg^9-bradykinin while the B_1 receptor has the opposite order of potency. Examples of B_2 responsive tissues typically used in assay are the rat uterus, cat ileum, and dog carotid artery. Tissues with B_1 properties are the rabbit aorta and dog vena cava (14).

The specificity of the kinin binding site is demonstrated by the inability of unrelated peptides to compete for the high affinity binding. The inability of angiotensin I and SQ 20,881 to block binding suggests that ^{125}I-Tyr-bradykinin is not binding to kininase II. Kininase II is synonymous with angiotensin converting enzyme and has been described in brain (93, 94).

Saturation experiments suggest that there are two high affinity components in cultures with nanomolar binding constants which are distinct from the nonspecific site observed in biphasic competition curves. However, care must be taken in analyzing the lower affinity nanomolar component because it is not saturated by the experiments performed.The curvilinear plot is interesting in that it is nearly identical to a curvilinear Scatchard plot of kinin binding in bovine myometrium (25), but different from Scatchard analysis of kinin binding in guinea pig ileum (26) which suggested a single 5 nM site. Two possible explanations are differences in the tissue examined or

differences in the ligands used to perform the saturation experiments. Odya et al. (25) used the same iodinated ligand that was used in the present study to obtain biphasic Scatchard curves, whereas Innis et al. (26) used tritiated bradykinin. Noncooperative interactions with ^{125}I-Tyr-bradykinin that are not evident with ^{3}H-bradykinin are a possibility.

Reversibility experiments suggested a multi-component system which could be resolved into two dissociation rates with half-lives of 88 minutes and 17 hours. Kinetic analysis using association and dissociation rate constants derived from Figures 24 and 28 yield affinity constants (K_D) of 0.76 pM and 0.08 pM for the fast and slow dissociating sites, respectively. Since these values are markedly different from values derived by Scatchard analysis, the possibility that a portion of the ligand was being trapped either by internalization of ligand or by desensitized receptors in a super-high affinity state was considered.

When identical cultures were allowed to associate ^{125}I-Tyr-bradykinin for either two or ten hours at 4° C no difference in the rate of dissociation of ligand could be discerned. In the unlikely event that internalization was occuring at 4° C in these cells, a decrease in the amount dissociated might be expected with longer association times. Since this was not observed at the points assayed, it is doubtful that the slow dissociation observed is due to internalization. Longer association times might also be expected to increase the slow dissociating component if longer incubation times allowed a greater portion of the binding sites to become super-high affinity and thus

resistant to giving up their bound ligand. While the results of prolonged association and dissociation may suggest that a conversion to super-high affinity states was not occuring, the possibility that a domain of receptors which had not already been maximally converted by two hours of association cannot be overlooked. Thus, eight additional hours of association would yield no additional conversion.

Discrepancy between kinetic analysis and Scatchard analysis of affinity constants has also been identified in prolactin recptors (170). Unlike the system described here, prolactin receptors demonstrate decreases in dissociation rate with prolonged association.

All cations show potent ability to decrease the specific binding of ^{125}I-Tyr-bradykinin. Divalent cations are substantially more potent than monovalent cations in decreasing specific binding. This ionic inhibition appears to be more than an ionic strength effect because divalent cations are not equipotent. Low manganese concentrations destroy greater than 90% of all specific binding. Sodium and calcium were eight-fold and ten-fold more potent, respectively, in reducing kinin binding in brain cells as compared to guinea pig ileum (26). Receptor binding for opiates (171) and α-adrenergic receptors (172) are regulated by sodium but the effect on these binding sites is much less potent. Potassium is considerably less potent than sodium in inhibiting opiate and α-adrenergic receptors.

The marked potency of divalent cations in inhibiting kinin binding is contrasted by its action on other receptors. Binding by agonists, but not by antagonists, for histamine H_1 (173), opiate (174), cholescystokinin (175) and α-adrenergic receptors (176) is enhanced by divalent cations.

Cultures with different neuron:glia ratios were compared to gather information about the cell type on which the kinin binding site resides. Young (six-day-old) and neuron-enriched cultures retain the high affinity binding site, but 11-day-old and glial-enriched cultures do not. Since the high affinity component was observed in cultures with a high neuron density and absent in cultures with very few or no neurons the results suggest that kinin binding sites were present primarily on neurons. That the effect observed was not due to loss of binding sites with increasing time in cultures is suggested by the fact that neuron-enriched cultures (Figure 30) were the same age as the 11-day-old cultures (Figure 29) which lacked high affinity sites. Some caution is warranted in interpreting these results because cells in enriched cultures were identified as "neurons" or "glia" only by morphology. Cell-specific antigens were not used to precisely define cell type or ratio. Consequently, the possibility exists that glial-enriched cultures may have contained cells which had dedifferentiated following several passages and were expressing a fibroblastic phenotype. However, recent observations that fibroblasts display specific, high affinity kinin binding (177) would argue against this possibility.

An increase in angiotensin II specific binding in SH rat brain (178) parallels a similar hyper-responsiveness to ivt angiotensin II in SH rats (169). Although a cause and effect relationship has not been demonstrated, an increase in B_{max} may explain hypersensitivity to angiotensin II in SH rats. Since hyper-responsiveness to ivt bradykinin in SH rats has been described (179), the possibility of a correlative alteration in kinin binding in cultures of SH rat brain was considered.

In limited studies SH rat brain cultures demonstrated a consistent increase in K_D of the higher affinity site. No consistent alteration in B_{max} could be found.

This study is the first report of kinin specific binding sites in brain tissue. Care must be taken in associating any binding data from rat brain cultures with physiological data from in vivo experiments in adult animals. The binding site in vivo may not be identical to an in vivo site, and these cultures were prepared from neonatal rats, not adults. Maturation may affect the disposition of kinin binding sites. Nevertheless, high levels of nonspecific binding and degradation in homogenates of adult tissue made investigation of binding in this preparation untenable. Brain cell culture minimizes these problems, and provides a functional system in which the physiology of kinin action may be studied.

CHAPTER VI
GENERAL SUMMARY

The evidence for kinin action in the brain, and the ability of kinins to bind to specific recognition sites in brain tissue can be reviewed within the context of the criteria previously described (p. 17) for the determination of kinins as neurotransmitters or neuromodulators.

Specific Kinin Recognition Sites

The initial step in neurotransmitter-receptor interaction can be studied in vitro with an appropriate radioactive ligand. The hypothesis that kinin binding sites represent physiologically relevent receptors can be supported by the fulfillment of three requirements: saturability, specificity and distribution. Chapter V addressed these requirements.

Saturability

Kinin binding sites saturate with a nanomolar affinity observed for other putative neuropeptides. Scatchard analysis indicates two components. The primary component was saturated and, consequently, well characterized. The secondary component from Scatchard analysis was not saturated and, therefore, its K_D and B_{max} values should be taken as estimates. A third component, described in competition experiments, appeared to be nonsaturable and probably represented nonspecific binding to the culture dish. The maximum number of binding sites is within a range expected for neurotransmitters.

When the K_D is determined as the ratio of dissociation rate constant (k_{-1}) and association rate constant (k_1) the values obtained are 10^4-fold lower than those calculated from Scatchard analysis. Since the association rate was in the range expected for a nanomolar affinity receptor the discrepancy in K_D is probably due to the extremely slow dissociation of the iodinated kinin from its receptor.

Specificity

Kinin analogues that are effective in mimicking the actions of bradykinin compete for binding at low concentrations. Des-Arg^9-bradykinin and unrelated peptides do not compete. The order of potency for competing kinin analogues suggests that the kinin binding site observed in brain cell culture is a B_2 binding site similar to that found in nonvascular smooth muscle and most vascular beds.

Distribution

Unless discrete dissections are performed, brain cell culture does not provide information on the regional distribution of binding sites. Techniques are available, however, to enrich the glial or neuronal components in brain cell cultures, and experiments in these two types of cultures can help distinguish the primary cell types on which a receptor resides. The observation that high affinity binding resides within neuron-enriched but not glial-enriched cultures suggests that kinin binding sites are primarily contained on neurons. Identification of kinin binding sites in specific brain regions could be performed by regional dissection or autoradiography, but will probably be forestalled until the overwhelming amount of nonspecific binding observed in whole

brain can be reduced. Autoradiography in brain cell culture would enhance the evidence indicating that kinin binding sites are located on neurons.

Other requirements

Kinin binding in brain cell culture also fulfills the requirements of tissue linearity, and ion and pH dependence. The stability of the ligand following exposure to brain cells was ascertained by chromatography.

Biological responsiveness from the kinin binding sites was not ascertained in the studies with cultures. The presence of a response is an important criterion in attempting to determine whether or not a binding site is relevant to a cell's function.

Exogenous Administration of Kinins

This criterion involves two important issues: localization of the effect and appropriate dose. The central kinin pressor response was localized to the ventral third ventricle by ventricular obstruction. Contrary to lesion experiments, ventricular plugs do not appear to cause gross anatomical damage. Ventricular plugging may be a more appropriate experimental approach when studying substances that can elicit their biological effects through the ventricular surface.

A dose-response relationship to the central kinin pressor response has been developed. The maximal pressor response requires 1-5 μg of bradykinin ivt. This dose appears high relative to other ventricularly effective peptides, e.g., angiotensin II. However, The question of appropriate dose is a complex one when observing the effects of ivt injections in the intact organism. Access to receptor sites and

variable rates of catabolism can tremendously decrease viability of any drug injected into the brain. The kinin specific enzyme kininase II (angiotensin converting enzyme) is concentrated in the choroid plexus lining the brain ventricles, and probably degrades a majority of bradykinin injected into the lateral ventricles before it reaches the site of action. Consequently, no distinction can be made between physiological concentrations and the pharmacological concentrations of kinins needed to elicit a response.

Peptide Inactivation

Because peptides in general can be degraded by numerous proteases this criterion is of minor importance by itself in helping establish kinins as potential neurotransmitters or neuromodulators. However, inhibition of the intense kinin degrading activity observed in both brain homogenate and brain cell culture was crucial to the establishment of a useful binding assay.

Conclusion

These data provide the first evidence for specific kinin binding sites in brain tissue and suggest that kinins interact with and through other brain systems to effect blood pressure. The results increase the understanding of kinin action in the central nervous system, and they enhance the current evidence suggesting that kinins are involved in neurotransmission.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality, as a dissertation for the degree of doctor of Philosophy.

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